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PURE CULTURES OF LARGE MONONUCLEAR LEUCOCYTES.

By ALEXIS CARREL, M.D., and ALBERT H. EBELING, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Plates 29 to 37.

(Received for publication, March 17, 1922.)

I.

INTRODUCTION.

Pure strains of cells are as necessary in the study of physiological problems as pure cultures of microorganisms in bacteriology. If leucocytes and lymphocytes could be kept for a long time in active condition in media of known composition, many fundamental facts concerning their normal function in the organism and their reactions against bacteria and foreign material would be elucidated. In 1920, several attempts were made in this laboratory to obtain a pure culture of large mononuclear leucocytes. Finally, a strain was isolated from the blood of a hen in January, 1921, by one of us, and maintained in active condition for nearly 3 months. Since then, other strains have been cultivated in the same manner. The purpose of this paper is to describe the characteristics of the pure cultures, the morphological changes of the cells, and their response to certain modifications in the composition of the culture medium.

II.

Technique.

1. Preparation of the Cells.—The blood of an adult chicken which had fasted for 24 hours was taken in cold paraffined tubes through an oiled cannula or syringe and centrifuged at high speed for 10 minutes. After removal of the plasma, a few drops of diluted embryonic tissue juice were placed at the surface of the buffy coat of

leucocytes. 15 minutes later, the coagulum containing the white cells could be removed and placed in a watch-glass with a small amount of Ringer solution. Generally, the film separated into clumps from which almost all the red cells could be washed away. Small fragments were selected and taken from the fluid with the point of a cataract knife.

2. Preparation of the Medium.—Several different media were used. In some experiments, no embryonic tissue juice was used and the medium was composed of plasma alone, or of plasma and Tyrode solution. In other experiments, the medium consisted of two volumes of plasma and one volume of chick embryo juice, or of one volume of plasma, one volume of chick embryo juice, and two volumes of Tyrode solution. Sometimes 20 per cent fibrinogen suspension was substituted for the plasma. In order to prevent the contamination of the white cells by fibroblasts or other cells, the tissue juice was diluted with Tyrode solution and centrifuged for a long time. After the mixture of tissue juice and plasma had been made, the culture medium was examined microscopically and the presence of any contaminating cells could be detected easily. In other cases, the tissue extract, before being used, was kept in cold storage for several weeks until the contaminating cells were dead.

3. Preparation of the Cultures.—The culture medium was placed on a cover-glass of mica, and a small fragment of the leucocyte film was embedded in it before coagulation. A certain relation should exist between the number of white blood corpuscles and the volume of the medium. If the leucocytes were too few in number or if the medium was overcrowded by the cells, the result was negative. When the culture was properly prepared, a great many ameboid cells were seen in the medium after 24 hours. Then the original fragment, which still contained a large number of cells, and the invaded area of the coagulum were divided into several parts and washed in Ringer solution. The section of the coagulum had to be made with greater care than in cultures of fibroblasts. If the knife was not very sharp and the edges of the coagulum were crushed or slightly folded, the cells could not migrate into the new medium. They multiplied in the old coagulum and died. After the first passage, the transfer into a fresh medium was made every 48 hours and even every 3rd, 4th,

or 5th day. The cultures were examined while living, and drawings and photographs were made of the cells. Some of them were stained with neutral red in concentrations varying from 1:10,000 to 1:40,000. A few cultures were fixed in warm Ringer solution containing 4 per cent of formalin, and stained with hematoxylin-eosin, Giemsa's stain, eosin and methylene blue, eosin-azure, and azure.

III.

RESULTS.

The first strain of large mononuclear leucocytes was kept in a condition of active life for almost 3 months. Some other strains were used for shorter periods of time.

1. Migration and Multiplication of the Cells.—After 24 hours, the fragment of coagulum containing the white blood corpuscles was surrounded by a very large number of cells which sometimes invaded the entire area of the medium (Fig. 1). The outer zone was made up of small ameboid cells. In fixed and stained cultures they appeared as polymorphonuclear leucocytes which are pseudo-eosinophilic. The inner zone consisted chiefly of larger ameboid cells. Some of them were very large and more or less elongated and branched, with reticular pseudopods. The others were smaller, more rounded in form, and emitted active filiform or lobar pseudopods. In the preparations stained with eosin-azure, these cells contained a darkly stained nucleus and some showed blue granules in their cytoplasm. They were lymphocytes and large mononuclear leucocytes. Some polymorphonuclear cells were also present in the inner zone. After 24 hours, fragments of coagulum containing the ameboid cells were extirpated from the cultures and placed in a new medium. The cells migrated almost immediately from the old into the new coagulum. They consisted of polymorphonuclear leucocytes, lymphocytes, and large mononuclears, but the number of polymorphonuclear leucocytes had markedly decreased (Fig. 2). If the cells were too closely packed in the old coagulum, their migration into the new medium was slow and death often occurred. After the third or fourth passage, no granular leucocytes remained in the cultures, which were composed merely of lymphocytes and large mononuclear cells. Later, the lymphocytes disappeared also (Fig. 3).

The activity of the cultures was irregular. The migration of the cells (Figs. 4 to 6) from the edges of the old coagulum started from the 1st to the 4th day. When a sharp section of the coagulum was made in the zone of the migrating cells, the medium was invaded sooner. During the first 24 hours, the cells multiplied in the old coagulum and accumulated in large numbers at the periphery where they sometimes formed masses of amorphous material. Later, a few ameboid cells grew from the edges (Figs. 4 to 6) and more or less suddenly toward the 3rd day a large number of them invaded the new medium. In other cases, the invasion of the medium began on the 1st day. The cells were usually disposed in chains which followed almost parallel paths into the new medium on several different planes, but every chain was laterally isolated from the others. When the growing masses were very dense and the cells in contact, the cultures generally died. Often chains of independent cells migrated far into the new medium. This phenomenon occurred generally after 2, 3, or 4 days, according to the technique used, the composition of the medium, and the previous activity of the cells. The multiplication of the cells was slow. However, a few mitotic figures were observed (Figs. 7 and 8). A normal culture was composed of a thick crown of elongated and branched cells, while the central part of the coagulum was practically free of cells. When the old coagulum contracted, or when the crushing or folding of its edges prevented migration in a measure, living cells and apparently dead material were observed in the center of the culture.

The general appearance of the cultures was strikingly different from that of a fibroblast culture. The cells had no tendency to form a tissue (Figs. 2 and 3), but always remained isolated. If they happened to be packed together, they died. When colonies of leucocytes were grown in Gabritschewski dishes, their edges showed no tendency to unite (Fig. 9).

The large mononuclear leucocytes invading the culture medium were almost uniform in shape, elongated or branched, and of irregular form. Their posterior end was generally rounded, while the anterior end emitted very active reticular pseudopods (Figs. 4 to 6). The nucleus was darkly stained by azure while the cytoplasm was clear. In the living cultures stained with neutral red, the cells showed a

segregation apparatus consisting of spherical bodies, often as large and even larger than the nucleus. After each passage, chains of cells of the same type, more or less densely packed, again invaded the medium. But under the influence of many technical conditions, and especially the composition of the culture medium, their rate of proliferation was liable to decrease. The two first strains which were isolated in January, 1921, lost their activity at the beginning of April and died soon afterwards. Another is still active after 3 months of life *in vitro*.

2. *Differentiation of the Large Mononuclear Leucocytes.*—In actively growing cultures, the appearance of the cells remained uniform. The strains were composed of ameboid cells, with more or less irregular darkly stained nuclei, and lobar, filiform, or reticular pseudopods (Figs. 5 and 6) which wandered from the edges of the old coagulum into the new medium. However, important morphological changes sometimes occurred. They were observed for the first time in December, 1920. Leucocytes had been taken from the blood of a hen and cultivated in homogenic plasma without the addition of any tissue juice. The coagulum was soon invaded by a large number of ameboid cells, which wandered through it for 2 days. Some of the cells agglutinated in clumps. At the periphery of, or within the clumps, a few cells modified their shape. The pseudopods disappeared and were replaced by sharp processes. On the 3rd day, fusiform and stellate cells resembling fibroblasts were seen in some of the clumps. There was no possible contamination of the culture by fibroblasts from another source because the cells had been taken from the blood and no tissue juice had been added to the plasma composing the medium.

The same phenomenon (Fig. 10 *a* and *b*) was observed subsequently in many cultures. It occurred generally when the migration and the multiplication of the lymphocytes were not very active. The transformation of the cells started in the old coagulum and its origin was multicentral. The cells which showed the first evidence of a change were the elongated lymphocytes with clear cytoplasm and reticular pseudopods. The central end of the cell lost its pseudopods and became a pointed and immobile process, while the peripheral end was still actively ameboid. These transition forms were apparently half fibroblasts and half ameboid cells. Sometimes the nucleus of an

ameboid cell lost its character of being darkly stained by azure, and became pale and oblong with two nucleoli like that of a typical fibroblast. Similar changes occurred in polygonal and branched cells which showed simultaneously fixed processes and reticular pseudopods. At the same time, typical macrophages (Fig. 11*b*) were seen at the periphery of the culture and sometimes in the immediate vicinity of the transition forms and the fibroblasts (Figs. 10*b* and 11*a*). The fibroblasts appeared often near the amorphous masses resulting from the accumulation of lymphocytes on the edges of the old coagulum. Pointed processes seemed to grow from this apparently necrotic material, and after a few hours, fusiform cells with slender processes could be seen. In a few cultures, the fibroblasts migrated from the old coagulum into the new medium where ameboid cells were still wandering (Fig. 10*b*). They were spindle-shaped or stellate cells with an outline not sharply defined, anastomotic processes, pale homogeneous cytoplasm, large oval nuclei, and generally two nucleoli (Fig. 12*a* to *d*). Of four cells united in a chain, two resembled spindle-shaped fibroblasts, the third was half fibroblast and half ameboid, and the fourth was a large mononuclear leucocyte. In this case it seemed that a chain of large mononuclears was being changed into fibroblasts, the mutation beginning in the central part of the coagulum. In other cultures, several fibroblasts were united by long processes in a reticulum.

Cultures which contained large mononuclears, transition forms, and cells resembling fibroblasts were stained with a weak solution of neutral red. The ameboid cells displayed large red granules, which sometimes filled the cytoplasm almost completely. Among the transition cells, some showed a large segregation apparatus and others small red granules. The fibroblastic forms presented very much finer granules, and some were completely free of them. In the latter case, the nucleus was oblong and clear, contained two nucleoli, and looked like the nucleus of a fibroblast. At the same time that their appearance was modified, the behavior of the cells changed. Instead of remaining isolated or forming a chain, they became able to unite sidewise with other cells and showed a tendency to form a tissue, while lymphocytes lived like isolated elements. The cells were acquiring not only a new morphological appearance but also the

property of associating with homologous cells in the manner of fibroblasts.

3. Action of Embryonic Tissue Juice and Serum on the Activity of the Cells.—It seemed probable that lymphocytes and large mononuclear leucocytes would respond to the presence of embryonic tissue juice and serum in the medium as do fibroblasts. But the sensitiveness of their reaction had to be investigated.

(*a*) In the experiments in which the action of embryonic tissue juice was tested, two kinds of media were used. The first was composed of 33.3 per cent normal plasma and 66.6 per cent of a mixture in different concentrations of embryonic tissue juice and Tyrode solution. The second was made of fibrinogen suspension, serum, Tyrode solution, and embryonic tissue juice. Large mononuclears in a condition of low activity at the third or fourth passage were cultivated in media containing 2.5, 33.3, and 66.6 per cent tissue juice. In 2.5 per cent tissue juice, very little or no migration took place and death occurred after one or two passages. On the contrary, the medium containing 33.3 per cent tissue juice was invaded by a large number of lymphocytes. In 66.6 per cent tissue juice, the migration was still greater, but the cells died after a short time, possibly because they were too closely packed. Similar experiments were made with a medium composed of 20 per cent fibrinogen suspension and 10 per cent serum, the presence of serum being necessary to obtain a firm clot which would not dissolve secondarily. The action of 2.5 and 40 per cent embryonic tissue juice was compared. The number of migrating cells was very much larger in the medium containing 40 per cent tissue juice, but they died sooner than in the 2.5 per cent medium. It was evident that the cells were very sensitive to the influence of the embryonic tissue juice, and that its optimum concentration in the medium was about 30 per cent.

(*b*) The action of homologous serum was studied in media composed of 20 per cent fibrinogen suspension, 5 per cent tissue extract, and varied concentrations of Tyrode solution and serum. The cells were obtained from cultures at the third and fourth passages and cultivated in media containing 10 and 50 per cent serum. In the first medium, no migration took place. The cells located in the old coagulum became round, and very few showed active ameboid

movements. They died after the first passage. On the contrary, in the second medium the cells located in the old coagulum retained their activity and a large number migrated into the new medium. After they were transferred to fresh medium, they perpetuated themselves for several generations. Similar results were obtained in media containing 70 and 10 per cent serum. There was no doubt that serum had a marked inhibiting influence on the migration of lymphocytes and large mononuclear leucocytes.

IV.

DISCUSSION.

The cells growing in pure cultures were large mononuclear leucocytes of Ehrlich (endothelial leucocytes, monocytes of Naegeli, blood histiocytes of Aschoff and Kiyono, or hemomacrophages of Metchnikoff). The pseudo-eosinophilic and eosinophilic leucocytes were never observed to be present after five or six passages. The lymphocytes, that is the small and medium sized mononuclear cells of Ehrlich, were seen in large numbers during the 1st week and disappeared afterwards spontaneously. Possibly they were transformed into larger forms, as Maximoff[1] observed in his cultures of lymphoid tissue. Then the cultures were composed exclusively of large mononuclear leucocytes. These cells maintained their activity *in vitro* for nearly 3 months. However, their rate of multiplication was slower than that of fibroblasts. The medium was probably not the optimum one. It may also be supposed that they cannot grow in solid medium in dense masses as fibroblasts do. When they were packed in the coagulum, they usually died after a short time. The actively growing cultures showed a large number of chains of ameboid cells radiating from the original fragment through the medium. But the chains were not in contact laterally. It seemed that the cells created around themselves a field unfavorable to homologous cells. They always remained isolated, while fibroblasts and epithelial cells in pure cultures formed a tissue. The colonies of lymphocytes and fibroblasts when in contact assumed a strikingly different appearance. The edges of fibroblast colonies had a marked tendency to unite.

[1] Maximoff, A., *Compt. rend. Soc. biol.*, 1917, lxxx, 225.

In some cultures, the fibroblasts of a colony seemed to be attracted by the fibroblasts of the neighboring colony and a group of several colonies soon formed a continuous layer of connective tissue (Fig. 13 *a*). On the contrary, the peripheral edges of the leucocyte colonies never coalesced, even when growing in close contact (Fig. 13 *b*).

The large lymphocytes were more sensitive to all influences than the fibroblasts. The slightest error in technique brought about the death of the cultures. This peculiarity increased the difficulties of the experiments very much, because marked differences of growth were observed which were due merely to technical errors. However, the cells responded readily to physical and chemical modifications of the composition of the medium by changes in the rate of migration and in their morphology. As cells cultivated *in vitro* respond to an antigen by the production of an antibody,[2] the use of pure strains of macrophages may, therefore, become of interest in the study of immunity.

The large mononuclears became transformed into cells which assumed the appearance of fibroblasts. These cells were spindle-shaped or stellate, with long and slender processes uniting sometimes in a reticulum with those of other cells. When stained vitally with neutral red, they could be distinguished from the large mononuclears present in the same culture by the neutral red reaction of Evans.[3] The nucleus was similar to that of the fibroblasts. They had also acquired a tendency to tissue formation which lymphocytes do not possess. Besides the typical cells, there were many transition forms characterized by a nucleus less darkly stained and more oval, by the progressive substitution of sharp processes for the pseudopods, and by a decrease in the size of the segregation apparatus. The macrophages had evidently differentiated into fibroblasts. It is probable that Maximoff observed the beginning of a similar transformation in his cultures of lymphoid tissue.[1] He mentions that the large lymphocytes cultivated in hypotonic plasma and bone marrow extract underwent a modification. Their cytoplasm was larger and less basophilic, while the nucleus became paler.

[2] Carrel, A., and Ingebrigtsen, R., *J. Exp. Med.*, 1912, xv, 287.

[3] Evans, H. M., and Scott, K. J., *Carnegie Institution of Washington, Pub. No. 273, Contributions to Embryology*, 1921, x, 3.

The differentiation of large mononuclears into fibroblasts is merely the confirmation of previous observations. Long ago, Renaut[4] found that young connective tissue formed inside uterine cysts, completely lined with epithelial cells, and thought it derived from ameboid cells which had migrated through the epithelium. He considered that fixed connective tissue cells, endothelial cells, clasmatocytes, chromoblasts, vacuolar cells, and osteoblasts originated from an indifferent lymph cell. The transformation *in vitro* of connective tissue macrophages into fibroblasts came as a partial confirmation of this view. In 1912, pure cultures of ameboid cells from connective tissue became transformed into fibroblasts.[5] Cultures of peritoneal macrophages were observed by Maximoff[6] to undergo differentiation and to yield colonies of fibroblasts. Maximoff[6,7] never observed the transformation of polyblasts into fibroblasts, probably for technical reasons. Foot,[8] in cultivating white blood corpuscles of chicks, saw no real fibroblasts in the preparations. But in cultures of leucemic blood, Awrorow and Timofejewskij[9] found that the lymphocytes, in multiplying and undergoing differentiation, were converted into various forms—macrophages, giant cells, and even spindle-shaped and stellate connective tissue cells.

It is well known that the genetic relationship between the types of connective tissue cells is very close. Sabin[10] has shown lately that clasmatocytes and monocytes, that is large mononuclear leucocytes, are identical in origin. Their differentiation into fibroblasts is, therefore, a phenomenon which could easily be expected and was already considered as certain. Policard and Desplas[11] consider that fibroblasts derive from lymphocytes in granulating wounds. According to Dubreuil,[12] the large mononuclear leucocytes wander into and become fixed in the connective tissue, grow larger, and transform

[4] Renaut, J., Traité d'histologie pratique, Paris, 1893, i, pt. 2, 968.

[5] Carrel, A., *J. Exp. Med.*, 1912, xvi, 165.

[6] Maximoff, A., *Arch. Russ. Anat., Hist. et Embryol.*, 1916, i, 105.

[7] Maximoff, A., *Compt. rend. Soc. biol.*, 1917, lxxx, 237.

[8] Foot, N. C., *J. Exp. Med.*, 1913, xvii, 43.

[9] Awrorow, P. P., and Timofejewskij, A. D., *Virchows Arch. path. Anat.*, 1914, ccxvi, 184.

[10] Sabin, F. R., *Bull. Johns Hopkins Hosp.*, 1921, xxxii, 314.

[11] Policard, A., and Desplas, B., *Compt. rend. Soc. biol.*, 1917, lxxx, 745.

[12] Dubreuil, G., *Arch. anat. micr.*, 1913–14, xv, 53.

themselves into clasmatocytes or rhagiocrins. Then their segregation apparatus decreases, and they become fixed connective tissue cells. The differentiation *in vitro* of lymphocytes into large mononuclears observed by Maximoff,[7] and of the large mononuclears into fibroblasts recorded by us, is a confirmation of the ideas of Renaut and his school,[4,12] who consider the lymphocyte as the origin of all connective tissue cells. The transformation of large mononuclears into fibroblasts *in vitro* has more significance than the mere final demonstration of a phenomenon which was practically known. Thus, it shows that cell differentiation may occur *in vitro* under conditions which can easily be controlled. There is, therefore, a basis for the hope that the immediate cause of the change will be discovered.

The pure cultures of large lymphocytes were found to respond more readily than fibroblasts to the presence of embryonic tissue juice or serum in the medium. Lymphocytes and polymorphonuclear leucocytes displayed a similar sensitiveness to the same factors. This means that leucocytes are submitted to the influence of the two classes of substances which we found to be instrumental in increasing and inhibiting the rate of cell multiplication. The activating substances,[13] as is well known, are contained in embryonic tissues and also in muscle, epithelial cells, and white blood corpuscles of adult animals, in the aqueous extracts of these tissues, and probably in their secretions. Their presence in a culture medium greatly increases the activity of fibroblasts,[14] epithelial cells, and also leucocytes. If leucocytes could be activated *in vivo* by tissue juices as much as *in vitro*, important consequences should follow. The humors in which tissue juices or cell secretions are set free would acquire the power of increasing the activity of leucocytes, lymphocytes, and fibroblasts. Aqueous extract of inflamed connective tissue, and peritoneal exudates, containing white blood corpuscles, have been found to possess such a power.[15] The death of groups of cells within a tissue would also set free their juices and stimulate the activity of leucocytes and lymphocytes. Should white cells secrete more freely or undergo leucolysis, the substances which were set free would stimulate the

[13] Carrel, A., *J. Exp. Med.*, 1913, xvii, 14.

[14] Carrel, A., *J. Exp. Med.*, 1913, xviii, 287. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317.

[15] Carrel, A., unpublished experiments.

activity and multiplication of the macrophages and other cells. By this mechanism, the destruction of a number of leucocytes within the organism should automatically be followed by an increase in activity and by the multiplication of the remaining cells.

It is known that blood serum possesses the power of inhibiting the multiplication of homologous fibroblasts,[16] and that leucocytes respond still more readily than fibroblasts to the retarding influence of serum *in vitro*. Very likely, serum possesses a similar action *in vivo* because the number of leucocytes would increase indefinitely if serum were not endowed with this property. As a consequence of the sensitiveness of leucocytes to serum, these cells must be more active in fluids containing serum proteins under a lower concentration. The relative activity and migration of leucocytes in interstitial lymph, inflammatory exudates, and blood may depend in some measure on this property. It is also probable that the increase of the growth-inhibiting action of serum in the course of life determines a decrease in the activity of the white cells and modifies their secretions. Possibly this is one of the mechanisms by which the profound changes brought about by age in blood serum may be related to certain diseases of the period of senescence, such as cancer. On the other hand, it must be remembered that within the organism the leucocytes are placed in a very complex medium, and that their response to the action of serum and tissue juices may be modified and even prevented by other factors.

V.

CONCLUSIONS.

1. Pure strains of mononuclear leucocytes were isolated from the blood of adult chickens and kept in active condition for nearly 3 months.

2. The cultures were composed of large mononuclear leucocytes which migrated and proliferated *in vitro* at a slower rate than fibroblasts. The cells had no tendency to form a tissue, as do fibroblasts and epithelial cells. They were much less resistant than fibroblasts.

3. Differentiation of the large mononuclears into cells assuming the appearance of fibroblasts took place under certain conditions.

4. The activity of the large mononuclears was increased by embryonic tissue juice and inhibited by homologous serum.

[16] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599.

EXPLANATION OF PLATES.

PLATE 29.

FIG. 1. 24 hour culture of leucocytes taken from the blood. In the lower left corner is seen part of the original mass of cells. The coagulum is invaded by a large number of cells. The inner zpne is composed chiefly of lymphocytes and mononuclear leucocytes and the outer zone of polymorphonuclear leucocytes. The intermediate zone which appears as a darker ring is occupied by large mononuclear ameboid cells. × 62.

PLATE 30.

FIG. 2. Second passage. The culture is still composed of mononuclear and polymorphonuclear leucocytes. The inner zone contains irregularly shaped, more or less elongated, ameboid cells, and the outer zone polymorphonuclear leucocytes, the number of which has decreased considerably. × 133.

PLATE 31.

FIG. 3. Twelfth passage. Pure strain of large mononuclear leucocytes. The polymorphonuclear leucocytes have completely disappeared. × 240.

PLATE 32.

FIGS. 4 to 6. Large mononuclear cells, with active pseudopods. × 1,000.

FIGS. 7 and 8. Mitotic figures in a pure culture of large mononuclears. × 1,000.

PLATE 33.

FIG. 9. Colonies of leucocytes in a Gabritschewski dish, showing their lack of coalescence. × 2.5.

PLATE 34.

FIG. 10. Culture 26330, eighth passage. (*a*) Pure strain of mononuclear leucocytes undergoing differentiation. There are a number of mitoses scattered throughout the preparation. × 100. (*b*) Higher magnification of the same culture. × 250.

PLATE 35.

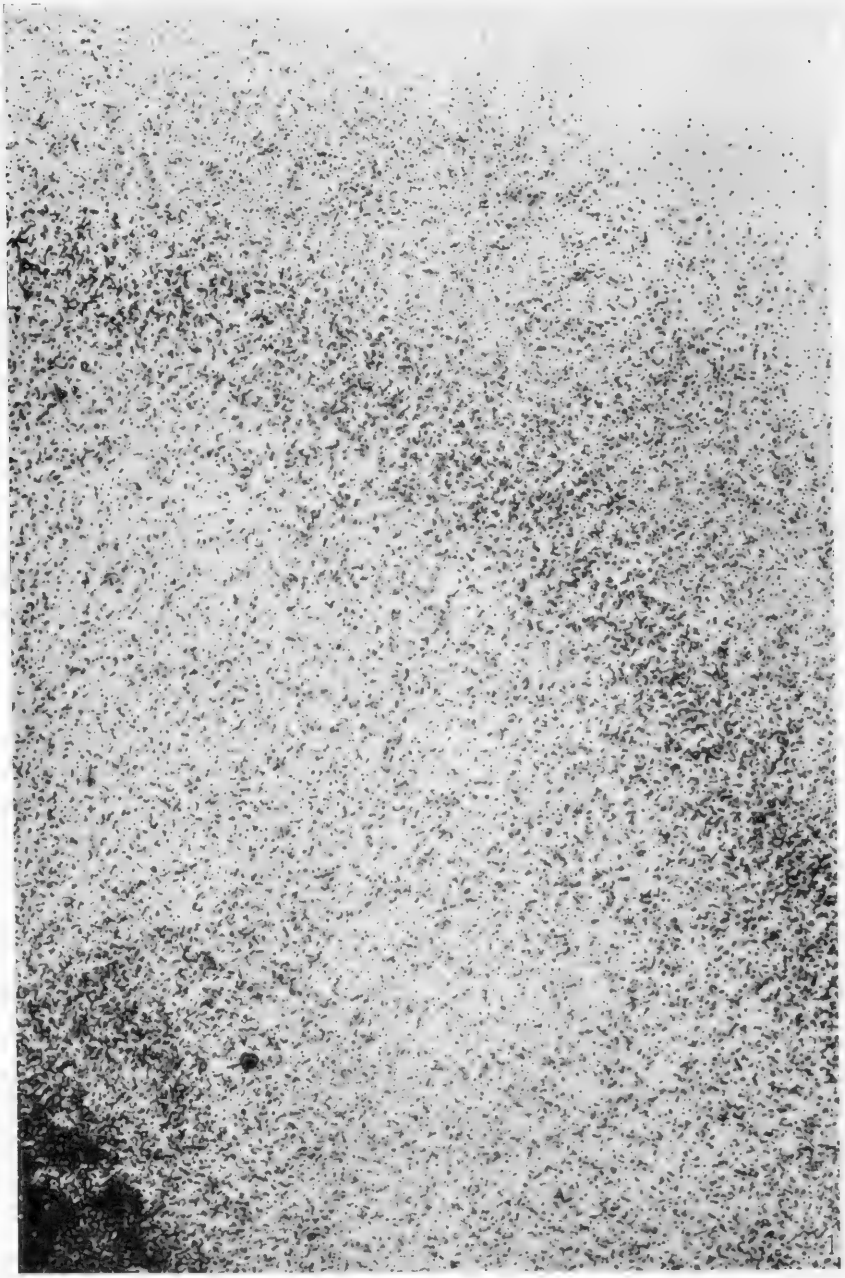
FIG. 11. Culture 26330, eighth passage. Higher magnification of two neighboring cells from the culture shown in Fig. 10. (*a*) Large mononuclear assuming the appearance of a fibroblast. × 1,000. (*b*) Large mononuclear, actively ameboid. × 1,000.

PLATE 36.

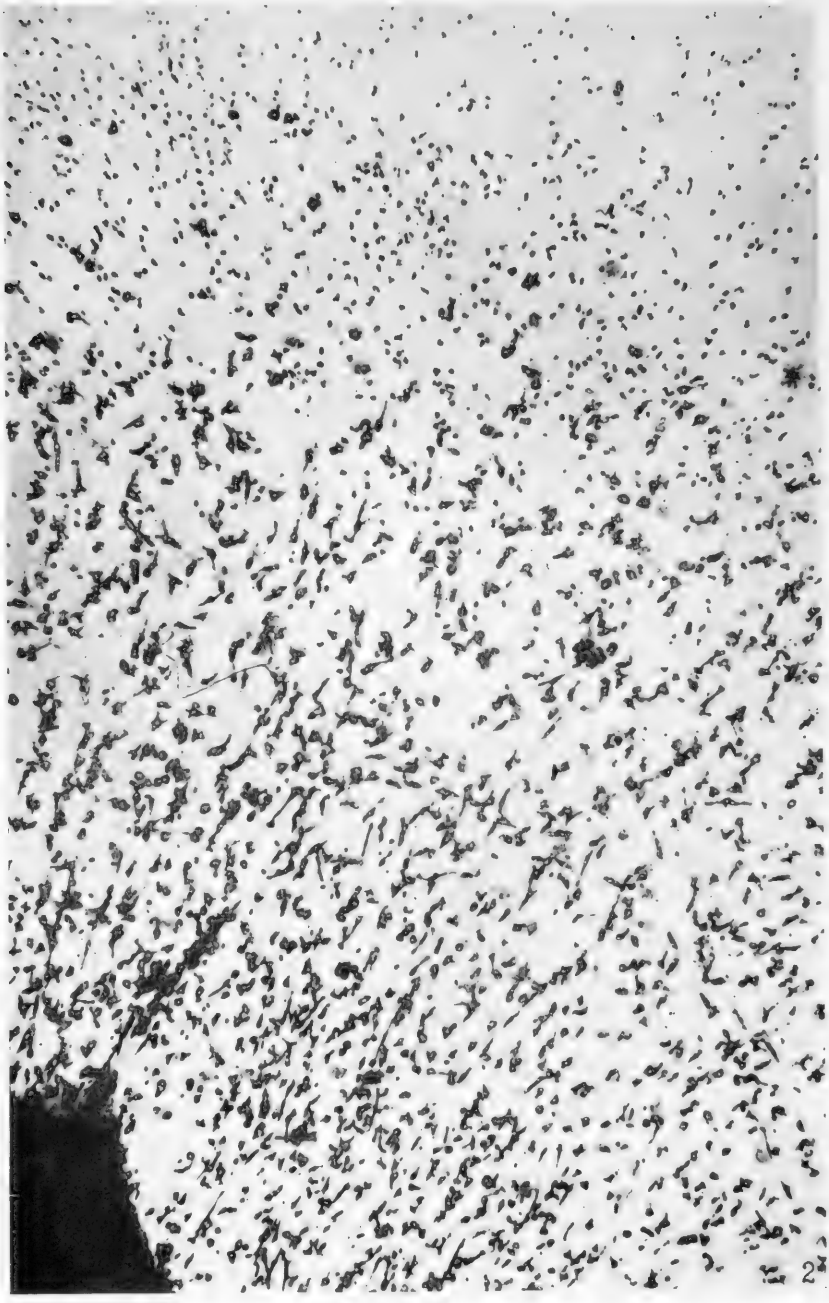
FIG. 12. (*a* to *d*) Large mononuclear leucocytes transformed into spindle-shaped and stellate forms. × 1,000.

PLATE 37.

FIG. 13. Semidiagrammatic drawing of colonies of (*a*) fibroblasts and (*b*) leucocytes. The colonies of fibroblasts have united at their edges.



(Carrel and Ebeling: Large mononuclear leucocytes.)



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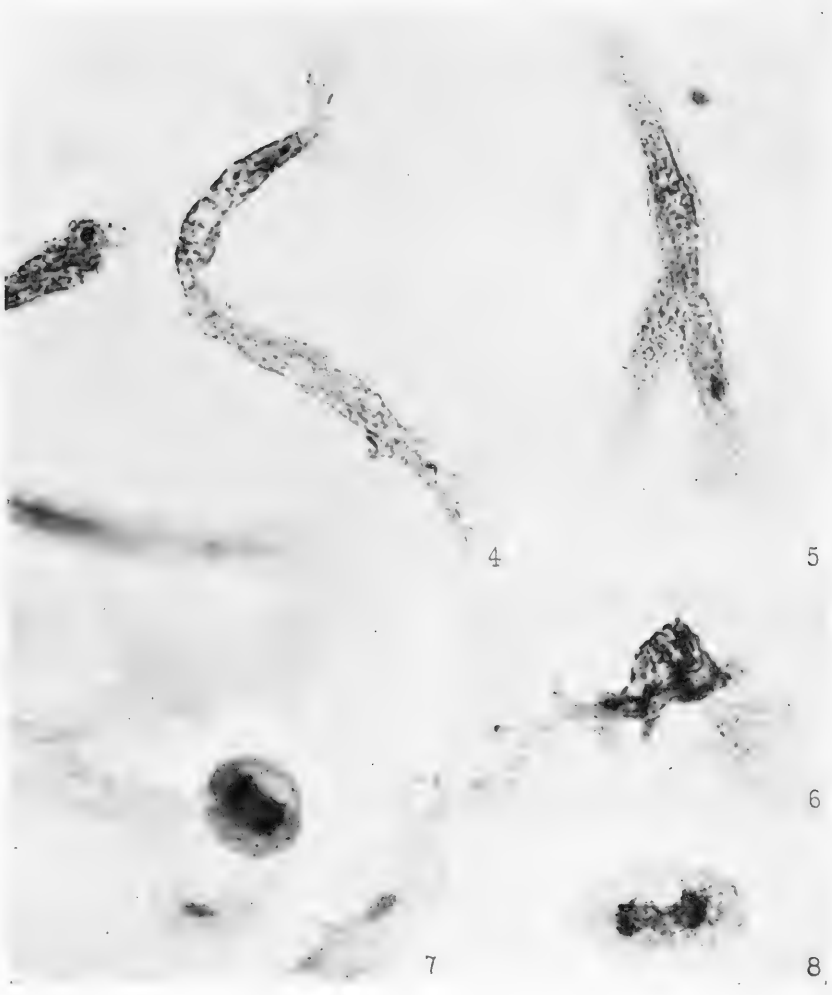

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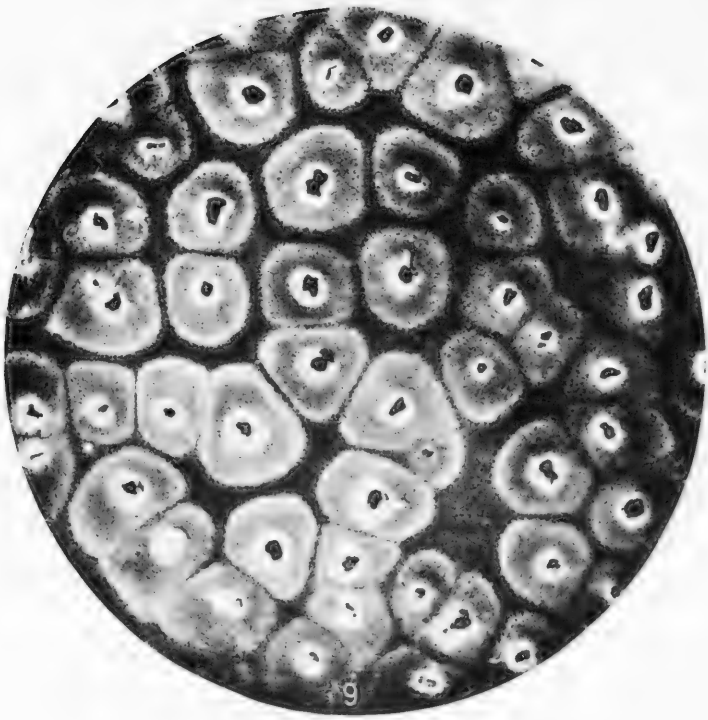
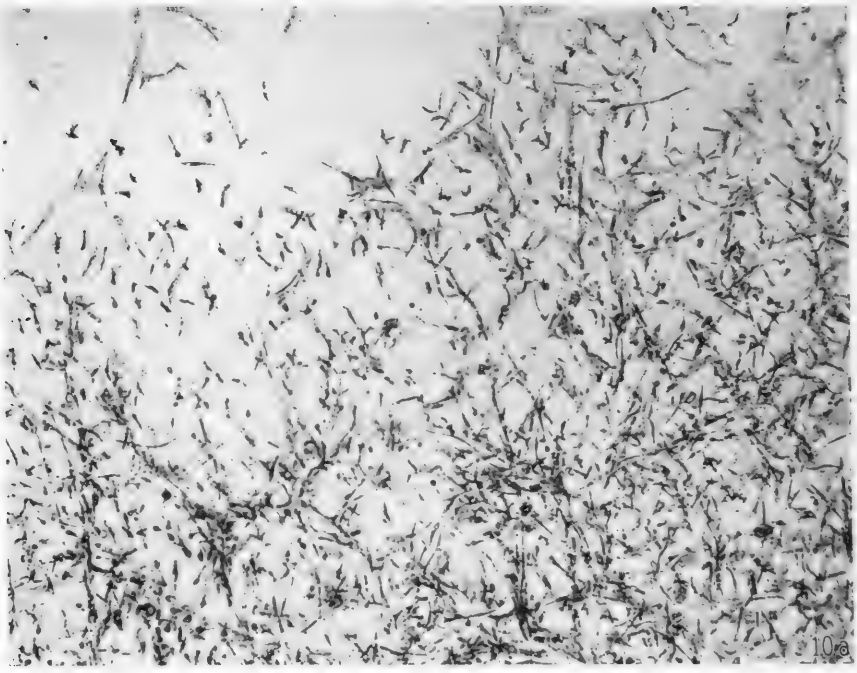
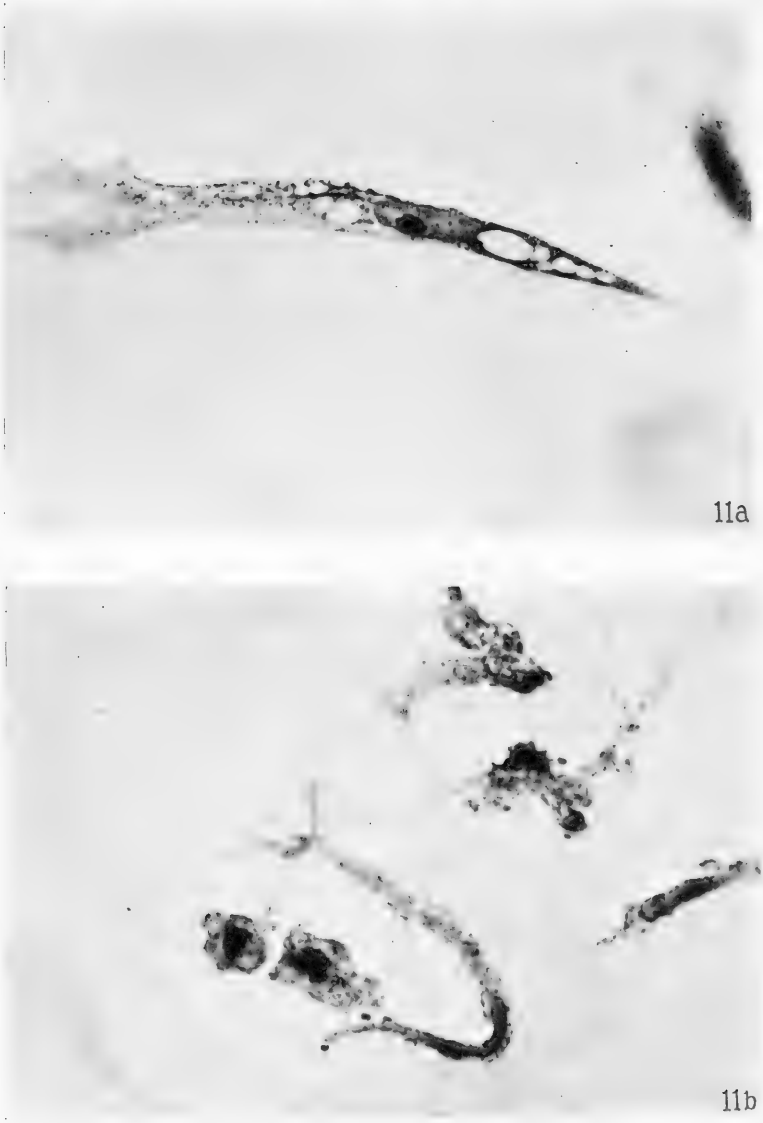


(Carrel and Ebeling: Large mononuclear leucocytes.)



(Carrel and Ebeling: Large mononuclear leucocytes.)



11a

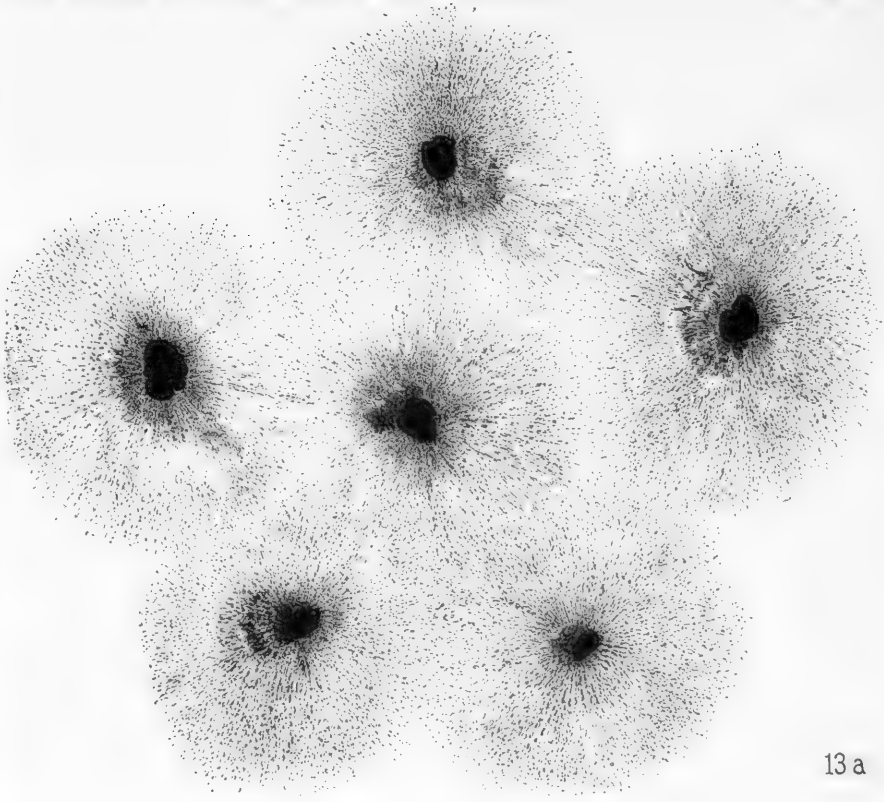
11b

(Carrel and Ebeling: Large mononuclear leucocytes.)

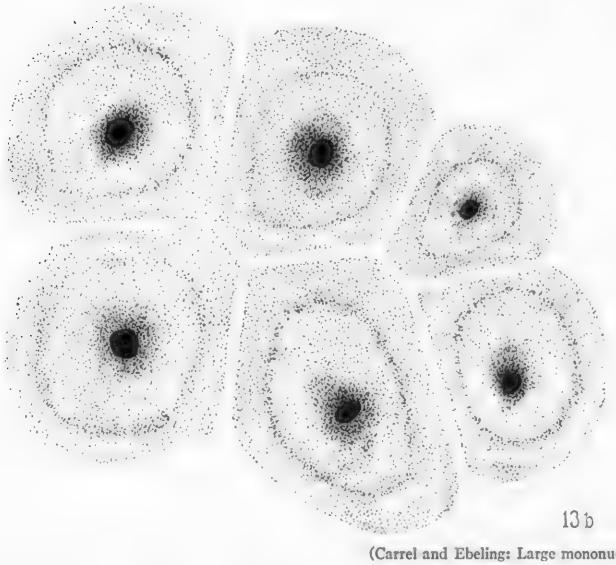
12a 12b

12c 12d

(Carrel and Ebeling: Large mononuclear leucocytes.)



13 a



13 b

(Carrel and Ebeling: Large mononuclear leucocytes.)

A PURE STRAIN OF CARTILAGE CELLS IN VITRO.

By ALBERT FISCHER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 38 TO 40.

(Received for publication, April 4, 1922.)

I.

INTRODUCTION.

No pure strain of cartilage has so far been isolated. However, the importance of working with pure cultures of the various types of tissue cells has been shown.[1] At present, only one method of obtaining a pure strain of a given type is known; namely, the isolation of tissues for cultivation from portions of the organism where they may be obtained free from any other tissue elements. In the eye of the chick embryo, we found an anatomical *locus electus* for obtaining pure cartilage. The sclera of the posterior hemisphere of the bulbus oculi in most birds and fish has a pars cartilago scleræ, and sometimes even a pars ossea. In sectioning the eye, it was noticed that free dissection of the cartilage could be made.

II.

EXPERIMENTAL.

1. Isolation of the Cartilage.—When the excised eye-bulb of the chick embryo is placed on its cornea, one may easily push aside the dark pigmented membrana nictitans with a small knife and an iris forceps; just below is seen a thin, white, semitransparent membrane. This is the cartilage. With the small forceps it may be lifted out. Immediately upon its removal, it retracts as does a drop of mucus, but when placed in a little Ringer solution, it spreads out as a thin membrane. In other respects it is not at all like mucus, but is very fragile and disintegrates easily.

[1] Fischer, A., *J. Exp. Med.*, 1922, xxxv, 367.

2. Technique and Description of the Cultures.—The culture medium is the same as that generally used for connective tissue cells and epithelium, consisting of equal volumes of adult chicken plasma and juice from chick embryos. The fragments of cartilage are placed on the surface of the coagulated plasma medium. Usually the growth does not begin in a promising way, but after three to five passages it becomes very active. If the piece of cartilage is embedded in the medium, as is done in cultivating fibroblasts no growth seems to take place. The fragment of cartilage remains apparently unchanged in shape in the same medium for many days, but by measuring the area of cartilage the day the culture is made and again after 3 or 4 days, or even longer, it is found to have increased in size. It is not certain whether this increase is due to a loosening of the tissue or to a veritable increase in the number of cells. When an attempt was made to transfer such a piece of cartilage into a new medium, after it had been embedded in the coagulum for 2 or 3 days, it proved impossible. The cartilage was so fragile that the slightest touch with the knife shattered it into innumerable pieces. If one is fortunate enough to secure a small piece on the knife and put it in Ringer solution, it spreads out very quickly, closely resembling a thin membrane of paraffin in water and being just as fragile. When it is placed in the new medium and examined under the microscope, thousands of aggregates and single cells are seen spread all over the medium. This picture is strikingly like that of a culture of yeast, especially because the cells are so small and apparently spherical. Therefore, no growth seems to take place from these colonies of cells after they are embedded in the coagulum.

An attempt was then made to place one of the small pieces of cartilage on the free surface of the clotted medium. As soon as this was done, the cells began migrating and progressively became more active and formed huge cultures. Cultivated in this way, the growth was very extensive and the cultures could be multiplied. These new cells grew in some ways like epithelium, forming thin, delicate membranes, while the individual cells were in close contact. The cultures were no longer fragile but coherent and easy to extirpate and transfer to a fresh medium.

When first examined, it seemed as if a new kind of tissue had formed which, in a measure, was true. The amorphous hyaline substance

had disappeared, and the cartilage cells grew and proliferated very actively side by side, without forming any of the hyaline substance. In order to study the details of this transformation, fresh cartilage was cultivated, embedded in the clot as well as on the free surface, and the cultures were fixed and stained in different stages. It was observed that the amorphous substance was loosened and fell to pieces, and the cartilage cells became free. The space around the cells could be seen to increase in size, and finally the hyaline substance liquefied completely (Fig. 1). The cartilage cells themselves were rather small, and the cytoplasm as well as the nucleus stained deeply. When the cartilage was embedded in the clot, the cells seemed unable to grow or to develop, unless they were in very close contact. Those cells which had been cultivated for some time in the clot were all very small. Nucleus and cytoplasm, the latter often very sparsely represented, were deeply stained (Figs. 2 and 9). The cells were about the size of small lymphocytes and the resemblance was rather striking.

From the surface-cultivated cartilage, the same process of breaking down the hyaline substance took place and a different type of cells migrated. All the transition forms could be studied here, from the small, lymphocyte-like cartilage cell to the much larger cell which finally characterized the active cartilage cultures. The same phenomenon could be observed as in Fig. 1, and the free cells gained in size rather quickly; *i.e.*, their cytoplasm was spread out (Figs. 3 and 4). Some resembled the cells in Fig. 5, having a deeply stained nucleus with the chromatin located at the periphery, and cytoplasm unstained and nearly as clear as glass. After further cultivation, the cells became spindle-shaped and the cytoplasm increased more and more, the cells finally appearing as in Figs. 6 and 10. The cytoplasm often contained large vacuoles. The nucleus was spherical and usually had one large nucleolus. The nucleus itself generally stained less deeply than the cytoplasm. The cells grew in close contact, forming thin membranes.

If, in this stage, the culture is transferred to the middle of the coagulum, it grows well and does not revert to the type of small cells.

III.

DISCUSSION.

Renaut and Dubreuil[2] came to the conclusion that the embryonic connective tissue originated from the lymphocytes. The various functions of the connective tissue determine the peculiarities of the amorphous substances excreted. In cartilage the collagen substance (connective tissue fibrillæ) is replaced by the hyaline substance. The transformation of lymphocytes into large mononuclears and polyblasts was observed by Maximoff,[3] and of large mononuclear lymphocytes into fibroblasts by Carrel and Ebeling.[4]

In our experiments, it was observed that a minute, lymphocyte-like cell type migrated out from the cartilage and became rapidly transformed into cells many times larger than the original ones. They did not produce any hyaline substance. When the size of the cells increased, great morphological and physiological changes occurred in them. The cytoplasm of the small cell type was very sparse, and the nucleus had a granular chromatin arranged in its periphery. Both nucleus and cytoplasm stained very deeply with methylene blue and azure blue. On the contrary, the cells which grew out on the surface of the coagulum were large and their cytoplasm was often filled with vacuoles. The nucleus generally had one large nucleolus and stained less deeply than the cytoplasm.

The segregation apparatus of Renaut when vitally stained with neutral red appears to be very similar to that of fibroblasts (Fig. 7). It was found that the initial growth of cartilage only took place on the free surface of the coagulum. When embedded in the coagulum, the cells were unable to adhere and no growth took place.

The strain of cartilage now under cultivation is more than 3 months old, grows very actively, and can be made to multiply. During this time it has been cultivated on the free surface of the coagulum which, at present, we find to be the best method for obtaining large cultures. Of all tissues hitherto cultivated *in vitro*, it resembles epithelium most, because of its characteristic growth in membranes (Fig. 8). The

[2] Renaut, J., and Dubreuil, G., *Compt. rend. Soc. biol.*, 1910, lxviii, 599.
[3] Maximoff, A., *Arch. Russ. Anat., Hist. et Embryol.*, 1916, i, 105.
[4] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxvi, 365.

cartilage liquefies the medium much more than epithelium and connective tissue cells do.

IV.

CONCLUSIONS.

1. A strain of cartilage cells, obtained from the pars cartilago scleræ of the eye of chick embryos, has been cultivated for more than 3 months *in vitro*.

2. The initial growth of the cartilage was possible only on the free surface of the coagulum.

3. The hyaline substance disappeared during cultivation *in vitro*. The succeeding stages of a transformation from small, lymphocyte-like cells into large, spindle-shaped cells were observed. The cartilage cells were spindle-shaped and grew in close contact, forming thin membranes. In surface-grown cartilage cells, the nucleus, usually containing one large nucleolus, stained less deeply than the cytoplasm.

4. The rate of growth of cartilage was slower than that of fibroblasts and epithelium. After cultivation on the surface of the coagulum, the cartilage cells could multiply even when embedded in the coagulum. But their growth was less extensive and uniform.

EXPLANATION OF PLATES.

PLATE 38.

FIG. 1. Cartilage after 48 hours incubation in plasma medium. The amorphous substances are broken up. The cartilage cells contain relatively little cytoplasm and the nucleus and cytoplasm are stained very deeply. Stained with azure blue.

FIG. 2. Types of isolated cells of cartilage from 2 day old cultures.

FIG. 3. Types of cartilage cells after escape from the amorphous substances.

FIG. 4. Types of cartilage cells cultivated on the surface of the medium.

FIG. 5. Cell types of cartilage found in cultures of fresh cartilage after 48 hours incubation. Nuclei deeply stained, with the granular chromatin arranged in the periphery. The cytoplasm is unstained and clear as glass.

FIG. 6. Cartilage cells growing on the surface of the medium.

PLATE 39.

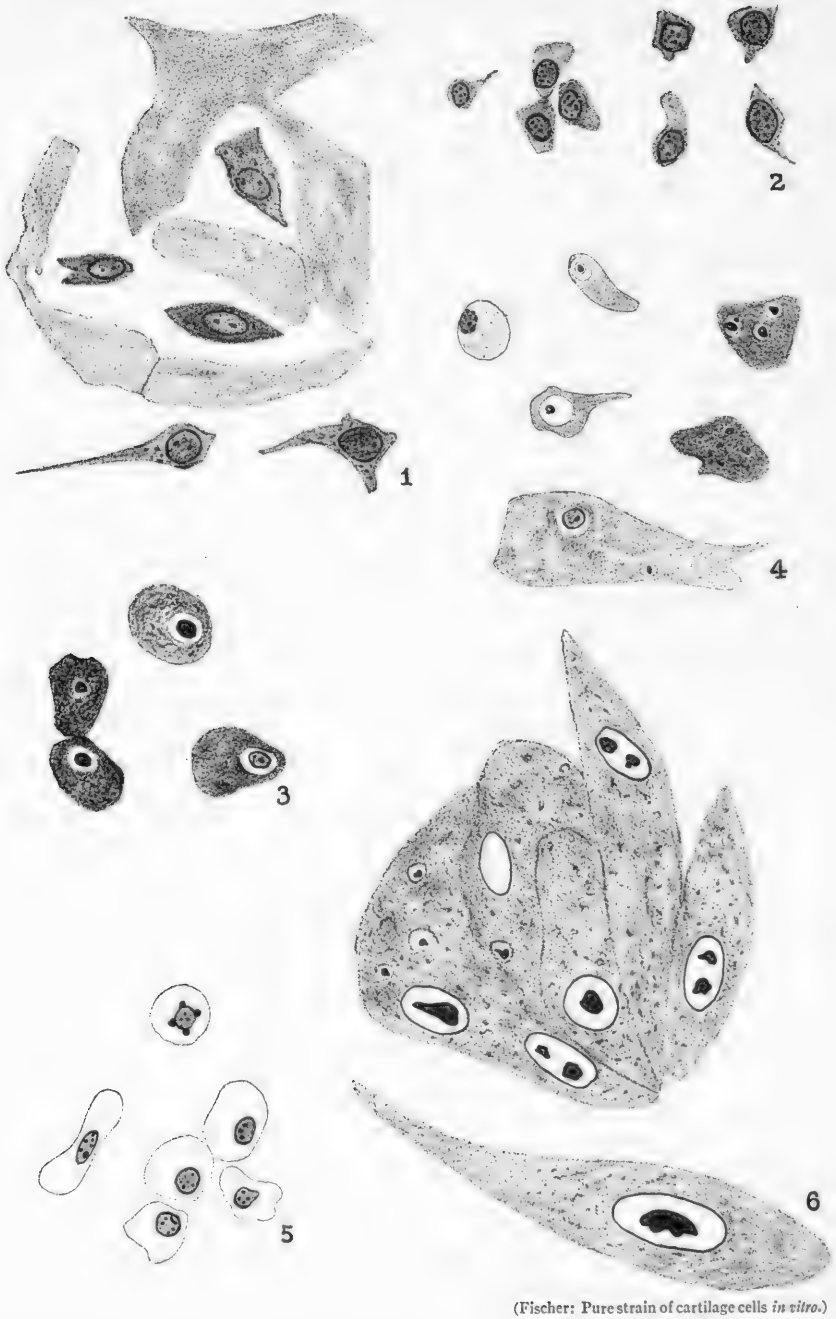
FIG. 7. The segregation apparatus of Renaut, vitally stained with neutral red. The cultures are 6 weeks old; 24 hours incubation.

FIG. 8. 2 month old culture of cartilage cells after 48 hours incubation. Stained with azure blue. × 60.

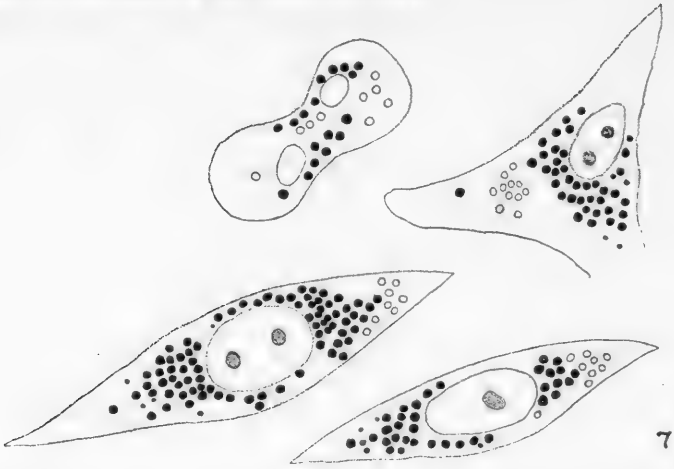
PLATE 40.

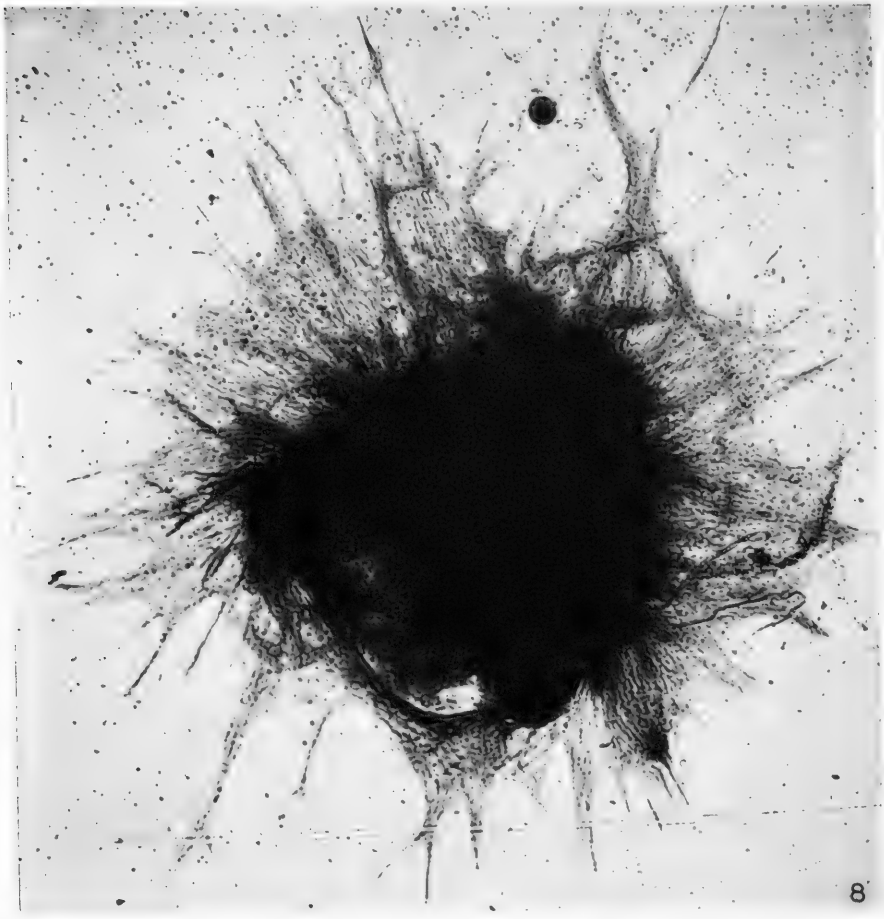
FIG. 9. Fresh cartilage cultivated on the surface of the plasma clot, showing the small types of cells just migrating on the medium. Stained with azure blue. × 1,000.

FIG. 10. Experiment 1634. Mitotic figures in cartilage cells. Stained with azure blue, × 1,000.

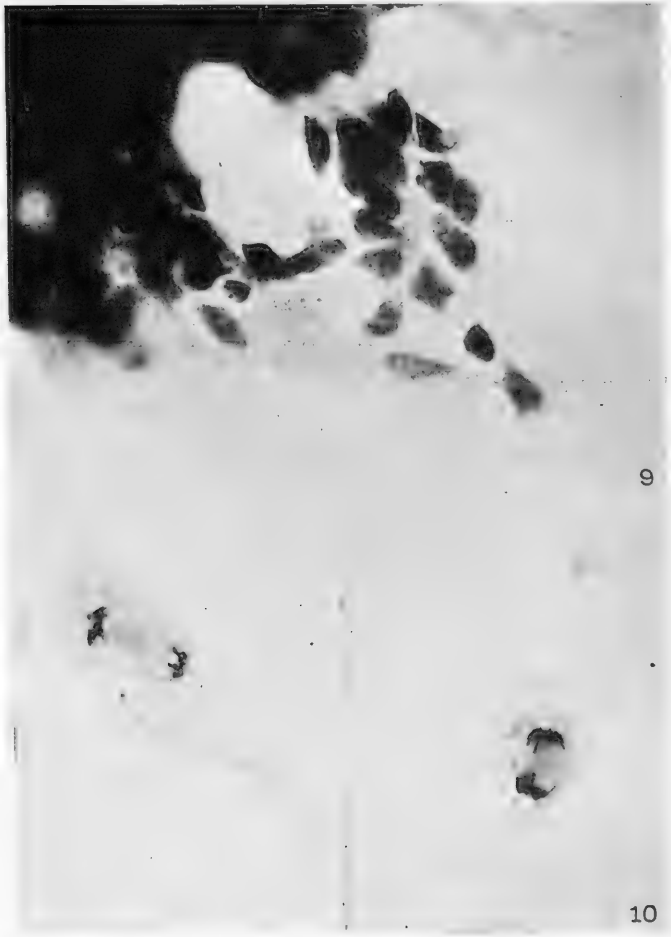


(Fischer: Pure strain of cartilage cells *in vitro*.)





(Fischer: Pure strain of cartilage cells *in vitro*.)



(Fischer: Pure strain of cartilage cells *in vitro*.)

GROWTH-PROMOTING FUNCTION OF LEUCOCYTES.

By ALEXIS CARREL, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, April 10, 1922.)

It is well known that the tissues of an adult animal remain capable of resuming the activity characteristic of youth. Even in extreme old age, wounds always heal, although cicatrization may require the formation of a large amount of new tissues. We are as yet ignorant of the mechanisms which cause cells at rest in a growth-inhibiting medium, such as the humors of an old animal, to proliferate again. The activity of a cell at a given instant is function of its activity at the preceding instant and of the concentration of certain substances in its medium.[1] Therefore, it is probable that tissues which have been in a resting condition for several years cannot grow again unless they receive the food material required by the cells for their multiplication. We know that certain substances contained in embryonic juice are endowed with the remarkable property of greatly activating the rate of cell proliferation *in vitro*.[1,2] Possibly such growth-promoting substances must be supplied to adult tissues when they cicatrize or regenerate. One of the sources of these substances may be the leucocytes which, remaining in the embryonic stage of development during the entire life of the organism, probably contain the growth-activating substances characteristic of embryonic tissues. The purpose of the experiments described in this article was to study the value of this hypothesis by ascertaining whether leucocytes contain and secrete growth-promoting substances, and whether tissues and exudates in which they accumulate acquire the power of activating cell proliferation.

[1] Carrel, A., *J. Exp. Med.*, 1913, xviii, 287.

[2] Carrel, A., *J. Exp. Med.*, 1913, xvii, 14. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317.

I.

Growth-Promoting Action of Leucocytic Extracts and Secretions.

Leucocytes were obtained from the blood of chickens 1 or 2 years old. After centrifugation, the leucocytes were removed from the surface of the red blood corpuscles, washed several times in Ringer solution, and placed in a small amount of distilled water. After 1 or more days in the ice box, the cell suspension was shaken with once

TABLE I.

Action of an Extract of Leucocytes on the Rate of Multiplication of Fibroblasts.

Experiment No.	Extract No.	Nature of tissue cultivated.	Culture No.	Width of ring of new tissue.		
				Leucocyte extract.	Embryo juice.	Ringer solution.
1	247	Embryonic heart.	247	4.0		2.0
2	247	" "	247	4.0		2.0
3	247	" "	247	5.5	6.0	
4	247	" "	247	5.0	6.0	
5	275	" "	276	5.0		2.5
6	275	" "	276	6.0		2.0
7	275	" "	288	3.5		2.0
8	275	" "	288	3.0		2.5
9	470	" "	490	4.0		2.5
10	470	" "	477	3.0		1.7
11	18488	" "	571	2.5	3.0	
12	18488	1685th passage fibroblasts.	571	4.0	3.0	
13	18488	1685th " "	575	3.0	3.0	
14	18488	1687th " "	587	5.0	6.0	
15	18488	First subculture from Experiment 14.	587	5.0	5.0	

or twice its volume of Ringer solution and centrifuged. The extracts were, therefore, more diluted than the embryonic juice generally used in tissue cultures. Their activity was tested against fragments of chick embryo heart and pure cultures of chicken fibroblasts. The medium was composed of one or two volumes of plasma and one volume of leucocytic extract. In the controls, the leucocytic extract was replaced by Ringer solution or by embryonic juice. The rate of growth was ascertained by measurements of the width of the ring of new tissue which had grown in 48 hours. Fourteen experiments were performed (Table I). Although the extracts were diluted, the

width of the growth was generally 100 per cent greater in the experiments than in the controls containing Ringer solution, and about the same as in embryonic tissue extract. It is evident that dead leucocytes set free substances which promote cell multiplication as do embryonic juices.

An investigation was then made as to whether living leucocytes may secrete growth-promoting substances such as are extracted from them after death. Long ago, growth-activating substances were found to be secreted by embryonic tissues *in vitro*.[1,2] Pulp of embryonic tissue was cultivated in plasma and Ringer solution. The experiments were incubated, while the controls were kept in cold storage. After 1 or 2 days, the serum was extracted from the coagulum and its activity tested against fibroblasts. The activating power of the fluid extracted from the cultures preserved in cold storage was lower than that of the cultures kept in the incubator. It appeared that the tissues growing actively for a short time had modified their medium by secreting in it growth-activating substances. Similar experiments were made with leucocytes. A medium composed of practically equal parts of chicken plasma and hypotonic Tyrode solution was spread on the surface of the cover of Gabritschewski dishes. About thirty fragments of a film of chicken leucocytes were placed in the medium. The control contained no leucocytes. Both dishes were incubated for 48 hours, and large colonies of leucocytes spread into the clot. Then the serum was extracted from the coagulum and tested against a pure strain of fibroblasts. The fluid from the medium which had contained leucocytes was markedly more active. It was evident that, under the conditions of the experiments, leucocytes secreted a substance which activated the rate of growth of fibroblasts.

II.

Growth-Promoting Action of Inflamed Connective Tissue Extracts and Peritoneal Exudates.

If leucocytes be capable of setting free growth-activating substances *in vivo* as well as *in vitro*, tissues and exudates where they accumulate must acquire the power of accelerating cell multiplication. Therefore, an attempt was made to ascertain whether inflamed connective tissue contains growth-promoting substances. It is known that extracts of connective tissue stimulate cell proliferation only slightly.[1]

However, control experiments were repeated. Subcutaneous connective tissue taken from a chicken was extracted with Ringer solution. The growth in the cultures containing the extract was about 25 per cent larger than in the controls containing only Ringer solution. In order that leucocytes could accumulate in the connective tissue, a focus of aseptic inflammation was produced by injecting a solution of dilute hydrochloric acid into fragments of sponge placed under the skin of chickens.[3] The connective tissue around the foreign bodies became markedly thickened, although no abscess developed. Fragments of inflamed connective tissue were then removed and cultivated in plasma in order to ascertain whether they contained living leucocytes. They were soon surrounded by a large ring of ameboid cells and after 24 hours the cultures looked almost like those of Rous sarcoma. The inflamed connective tissue was sliced into a pulp, extracted with a small amount of Ringer solution, and the extract tested against fibroblasts and embryonic heart. The medium was composed of one or two volumes of plasma and one volume of extract. The controls contained Ringer solution or embryonic tissue extract. Although the inflamed tissue extracts were dilute, the width of the new tissue was more than doubled by their presence (Table II). There was no doubt that inflamed connective tissue contained substances capable of increasing the rate of multiplication of fibroblasts.

Similar experiments were repeated with peritoneal exudates. Injections of staphylococcus suspension, dog red blood corpuscles, turpentine, or bouillon into the peritoneum of chickens did not determine the production of an exudate. However, pus obtained from a turpentine abscess in a dog was injected into the peritoneum of two chickens, and an opaque fluid, orange-yellow in color, was aspirated from the abdominal cavity 2 days later. This fluid contained a large number of white blood corpuscles. After centrifugation, it was tested against embryonic heart tissue and fibroblasts. In twelve experiments, the width of new tissue was 100 per cent larger than that obtained in Ringer solution and about equal to that in embryonic juice (Table III). It was then certain that a peritoneal exudate containing many leucocytes had acquired the power of stimulating cell multiplication.

[3] All operations were performed under ether anesthesia.

TABLE II.

Action of an Extract of Inflamed Connective Tissue on the Rate of Multiplication of Fibroblasts.

Experiment No.	Extract No.	Nature of tissue cultivated.	Culture No.	Width of ring of new tissue.		
				Inflamed tissue extract.	Embryo juice.	Ringer solution.
1	213	Embryonic heart.	258	1.0		0.5
2	213	" "	258	1.5		0.5
3	213	" "	258	1.5		0.5
4	213	" "	263	4.2		2.7
5	213	1650th passage fibroblasts.	261	7.0	5.0	
6	213	1650th " "	261	5.5	6.0	
7	213	Embryonic heart.	269	3.0		1.0
8	213	" "	269	3.0		1.5
9	213	" "	269	4.0		1.5
10	311	" "	322	3.0		2.0
11	328	" "	332	4.0		2.0
12	328	" "	332	4.0		2.0
13	339	" "	349	1.5		0.8
14	339	" "	350	3.0		0.8

TABLE III.

Action of Peritoneal Exudate on the Rate of Multiplication of Fibroblasts.

Experiment No.	Exudate No.	Nature of tissue cultivated.	Culture No.	Width of ring of new tissue.		
				Exudate.	Embryo juice.	Ringer solution.
1	844	1719th passage fibroblasts.	846	5.0	5.0	
2	844	1719th " "	846	3.0	4.0	
3	844	Embryonic heart.	852	2.0		1.0
4	844	" "	852	2.0		1.0
5	850	" "	858	2.0		0.8
6	850	" "	858	1.5		0.5
7	850	" "	858	3.5		0.5
8	850	" "	858	4.5		0.5
9	850	" "	864	2.5	2.0	
10	850	" "	864	1.75	2.0	
11	866	1727th passage fibroblasts.	852	2.0		1.0
12	866	1727th " "	852	2.0		1.0

III.

DISCUSSION AND SUMMARY.

Two main facts were brought to light by the preceding experiments: first, the presence of growth-activating substances in the leucocytes; second, the setting free of these substances in tissues and fluids where leucocytes accumulate. The existence of growth-promoting substances within the body of the leucocytes was to be expected. Leucocytes are embryonic cells and it is well known that embryonic tissues contain substances which stimulate cell proliferation. But the experiments gave a direct experimental proof of this fact. Then, during the whole life and even in extreme old age, there is a supply of growth-promoting substances within the organism which is potentially capable of restoring the activity of the resting cells. Embryonic tissue juice, as is known, can rejuvenate cells which have ceased to multiply *in vitro* and show evidences of degeneration.[1] If the growth-activating substances of leucocytes can be transferred *in vivo* to tissue cells, they may play a similar rôle.

Therefore, it was important to find out whether the growth-activating substances were set free either by the secretions of the living leucocytes or by diffusion from the body of the cells after they were injured or dead, and whether this phenomenon occurred actually *in vivo*. Indeed, the idea that leucocytes secrete substances necessary to normal physiological processes is by no means new. Long ago, Ranvier described the lymph cells as mobile unicellular glands,[4] and Renaut thought that their function was to bring to fixed cells the necessary food material.[5] This rôle of the leucocytes was considered by him as absolutely essential. According to his theory, differentiated cells could not live in the absence of the lymph cells, which supply them with the substances required for their activity. The presence of such substances in the leucocytes was shown by our experiments and the growth-activating power acquired by inflamed connective tissue demonstrated that the leucocytes could actually bring these substances to the fixed cells.

[4] Ranvier, cited by Renaut, J., Traité d'histologie pratique, Paris, 1889, i, pt. 1, 79.

[5] Renaut, J., Traité d'histologie pratique, Paris, 1889, i, pt. 1, 79, 94.

Under certain conditions, this property of the white cells of the blood may cause the resumption of the activity of tissues which are in a resting state. In the adult organism, the tissues have ceased to grow and the blood plasma has acquired marked inhibiting properties. But growth-promoting substances are still stored in leucocytes, glands, and muscle tissue. The leucocytes could supply fibroblasts or epithelial cells with the necessary food material if they were present where cell proliferation is needed. The existence of mechanisms causing leucocytes to invade tissues in need of repair is certain. The initiation of healing seems to depend on the coming of the leucocytes to the wounded tissue. When they are missing, as happens when the wound is protected from all external irritation, cicatrization is greatly delayed. On the contrary, when staphylococci, turpentine, and other irritants are applied at the surface of the wound, granulations appear after less than 48 hours.[6] These irritants, although different in nature, have the common characteristic of determining an inflammation of the tissues and the migration of leucocytes from the vessels to the surface of the wound. Possibly the white cells bring the substances which adult tissues require in order to cicatrize or regenerate. They would have the function of storing away the growth-promoting substances characteristic of embryonic tissues, and bringing them to the regions of the organism where they are needed.

IV.

CONCLUSIONS.

1. Leucocyte extracts, like embryonic tissue juice, possess the power of increasing the rate of multiplication of fibroblasts *in vitro*.

2. Leucocytes secrete substances *in vitro* which also promote cell multiplication.

3. Peritoneal exudate or connective tissue invaded by leucocytes acquires the power of increasing the rate of multiplication of fibroblasts.

4. Leucocytes are capable of bringing growth-activating substances to tissue cells. They may have the important function of promoting cell multiplication in the parts of the organism where they accumulate under certain conditions.

[6] Carrel, A., *J. Exp. Med.*, 1921, xxxiv, 425.

CULTURES OF ORGANIZED TISSUES.

By ALBERT FISCHER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 41 AND 42.

(Received for publication, May 31, 1922.)

It was shown by Thomson[1] that if a whole organ or a complete chick embryo 24 hours old was explanted to a culture medium, it increased markedly in size and no outgrowth of cells took place. If part of an organ, for instance a toe of a chick embryo, was explanted, an uncontrolled proliferation of cells began from the injured parts. When the basement membranes are injured, the cells begin growing out into the medium, whereas an uninjured organ continues to grow as a complete structure controlled by the laws of the organism. Thomson calls this "somatic growth." The increase of the organ stopped entirely after it reached a certain size. Possibly the cessation of growth was due to lack of absorption of the nutriment.

The experiments reported herein are in a way similar to those of Thomson. We used fragments of intestines from chick embryos which were about to hatch. At this period of embryonic development, the intestinal epithelium is assumed to possess its normal function.

I.

Technique.

The experiments were carried out in the following way. Chick embryos about 21 days old were taken out of the shell aseptically. The abdominal cavity was exposed and a fragment of the small intestine was extirpated and placed in Ringer solution. In some cases, the intestine was opened with a pair of fine scissors and a little strip was cut off and placed in the culture medium, which consisted of equal volumes of chick plasma and tissue juice. In other experi-

[1] Thomson, D., *Proc. Roy. Soc. Med.*, 1913–14, vii, Marcus Beck Lab. Rep., 71.

ments, the fragment of intestine was turned inside out, leaving the epithelium outside and the serosa inside the lumen. It was washed to free it from the meconial mucous secretions and transferred to the medium. After 48 hours incubation at 39°C., the culture chamber was opened and the fragment was picked out with the point of a cataract knife, or was aspirated in a pipette. Then it was washed for a minute or so, and transferred to a new medium. The small fragments of intestine brought about an extensive liquefaction of the clot, so that after 48 hours incubation, they were usually found floating in a cup-like excavation, surrounded with fluid.

II.

Description of the Cultures.

In some of the cultures, an outgrowth of connective tissue or epithelium from part of the fragment could be seen; in others, there was no uncontrolled growth whatsoever. After a few passages, the proliferation usually stopped and the epithelium could be seen growing all around the fragment, which was completely covered by epithelial cells after 48 hours. There was then no further uncontrolled growth. At this stage, the fragment had become spherical and its surface was as shiny as fresh mucous membrane. Under the microscope, it often appeared as a semitransparent body with a slightly denser central portion. In the early stage of life *in vitro*, numerous intestinal villi were visible and the individual cells of the cylindrical epithelium could easily be distinguished. Active peristaltic movements were observed around the free edge of the cuticula of epithelial cells. A broad, semicircular pseudopod was often observed to protrude from one cell, followed by a similar activity in the neighboring cells, whereupon the first one relaxed. It was also possible to perceive migration of the ameboid cells between the epithelial cells.

In many experiments, the active contraction of the intestinal muscles could be seen *in vitro*, even after a month's cultivation. These contractions appeared as slow as the peristaltic movement *in vivo* and were brought about by a slight cooling of the preparation.

The size of the spherical bodies, or "organisms," varied according to their age. In general, they tended to grow smaller as time went on.

When the tissue was transferred to a fresh medium, the body contracted markedly, due to cooling and irritation during the manipulation, but after incubation for an hour or two in the new medium, it relaxed and consequently increased in size. Shortly thereafter, liquefaction took place around the epithelium, which secreted a mucous fluid. In a few cases, the actual secretion could be seen to exude from the epithelial cells. At the same time, several ameboid cells could be seen wandering through the stomata of the epithelial coat. They usually deteriorated rapidly in the mucus which surrounded the fragment. In later states, the intestinal body became pellucid, and the coat of regular cubic epithelium could be studied thoroughly. Often it was possible to observe small appendicular cystic formations which appeared on the surface of the body within a few hours (Fig. 1).

III.

Histological Examination.

After a month's cultivation, the tissues were fixed in 2 per cent formol-Ringer solution, and sectioned in series. They were found in an excellent state of preservation. The cylindrical epithelium had grown all around the fragment (Fig. 2). The villi had disappeared and the surface of the body was very even and smooth. The epithelium in the deep Lieberkühn glands was well preserved in many cases, but did not seem to have any communication with the outside. The connective tissue cells had formed a stroma with uumerous fibrillæ. There were no evidences of uncontrolled growth, as the epithelial cells did not go beyond their natural border-line, and there was no intermixing of the different cells.

It was supposed that bodies such as this would be able to live in a fluid medium. Therefore, fragments of intestines were cultivated in the usual way in chick plasma and tissue juice until they were entirely coated with epithelium. Then they were placed on the bottom of hollow slides and 3 or 4 drops of tissue juice were added. At the end of about a month, they were fixed, sectioned, and stained. There was a marked difference between those cultivated in the fluid medium and those in the solid medium. In the fluid medium, the villi were perfectly preserved, and very little stroma was left. In the

center of the "organism" only a faint shadow of the stroma remained. Just under the lining epithelium which appeared to be normal, were a large number of epithelial cells in a more or less ameboid state. Under this layer, an empty space could be seen which had probably been filled with liquid, and finally toward the center of the body, a very thin, loose stroma with single connective tissue cells.

The cylindrical epithelium was very well preserved. Its free surface formed a continuous and solid cuticula. This contact between the epithelial cells was only perfect in their peripheral part. Between the external and the basal parts of the cells, free spaces could be seen, as if they had been formed under the pressure of a liquid within the body. The epithelial border of one which had been kept in a fluid medium for a long time is shown in Fig. 3.

IV.

DISCUSSION AND SUMMARY.

An artificial organism, if one may so term it, composed of a complex of tissues, was cultivated for a long period of time. Small fragments of intestine from chick embryos 20 to 21 days old were placed in a suitable medium. The epithelium proliferated and completely covered the fragment of intestine after 4 to 6 days. A small body was thus formed, round or oblong in shape, surrounded by cylindrical epithelium and containing epithelial, connective, and muscle tissues, endothelium, and ameboid cells. After a month's cultivation *in vitro*, no necrosis had occurred. Therefore, it may be assumed that, through the intestinal epithelium, the medium supplied the intestinal tissue with sufficient nourishment. No uncontrolled proliferation took place after the epithelium had surrounded the entire fragment.

The cultivation of complex tissues will facilitate the study of the interactions of the different cells under various conditions. In some experiments, pure cultures of epithelial cells were grafted into such an "organism" without difficulty. The growth of malignant cells could be studied in the same way. When the "organism" was placed in a fluid medium, the epithelium remained normal but the stroma disappeared. It seems that plasma played an important rôle in the maintenance of the tissues in their normal condition.

V.

CONCLUSIONS.

1. Fragments of small intestine from a 21 day old chick embryo, cultivated in plasma and tissue juice, became completely surrounded with cylindrical epithelium.

2. After a month's cultivation in plasma and tissue juice, the tissues composing the mass were normal. It would seem that the necessary food material was absorbed by the epithelium from the culture medium.

3. When these masses were cultivated in embryo juice without plasma, the intestinal villi remained normal, while the stroma, connective tissue cells, and their fibrillæ progressively disappeared, leaving an epithelial cyst.

EXPLANATION OF PLATES.

PLATE 41.

FIG. 1. Experiment 2107–24. Section of an intestinal "organism" cultivated in embryonic juice without plasma. The epithelium is normal. The stroma is very loose and the cells are sparsely represented. A cystic formation is seen at *A*. × 150.

FIG. 2. Experiment 1085–6. Section of an intestinal "organism" cultivated in plasma and embryonic juice for a month. × 200.

PLATE 42.

FIG. 3. Experiment 2107–22. Section of an intestinal "organism" cultivated in embryonic tissue juice for a month. The loose stroma and the cubic epithelium with the dilatated spaces between the individual cells may be seen. × 272.

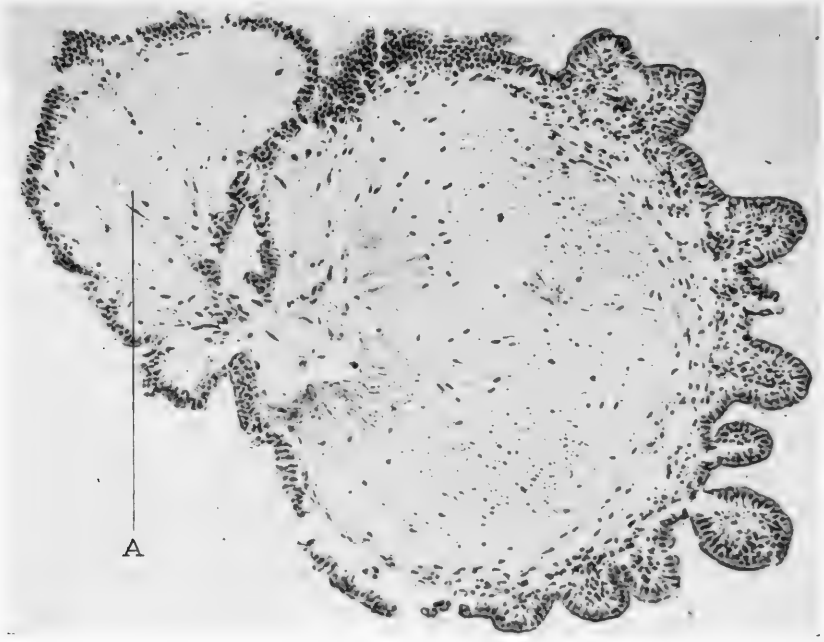


FIG. 1.

FIG. 2.

(Fischer: Cultures of organized tissues.)

FIG. 3.

(Fischer: Cultures of organized tissues.)

ACTION OF SHAKEN SERUM ON HOMOLOGOUS FIBROBLASTS.

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(Received for publication, June 1, 1922.)

The activity of homologous fibroblasts decreases after the serum composing their medium has been heated at 56° and 70°C., while under the same cultural conditions, the rate of migration of heterologous fibroblasts increases.[1] The better growth of heterologous tissue in heated serum is probably due to the destruction of alexin. But the cause of the decreased activity of homologous tissue is still unknown. It may be attributed to the disappearance of alexin or of some other factor which should have, in common with alexin, the property of being thermolabile. Therefore, an attempt was made to ascertain whether factors other than heat might bring about simultaneous changes in the inhibiting action of serum on homologous fibroblasts, and in its alexinic activity. The purpose of the experiments described in this article was to study the effect of serum inactivated by shaking, on the growth of fibroblasts.

I.

Action of Shaken Serum on Foreign Erythrocytes and on Homologous Fibroblasts.

Serum was obtained from plasma of chickens about 1 year old, sealed in Pyrex tubes, and shaken at low speed for periods varying from 1 to 8 hours. It became more or less opalescent and, after centrifugation, a slight precipitate settled at the bottom of the tube. The volume of the precipitate varied according to individual differences in the animals. It was never very large, and the variations of the refractive index of the serum before and after shaking were

[1] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 647.

always slight. There was practically no difference in the H ion concentration of the fluid. The lytic action of serum on foreign cells was ascertained with sheep corpuscles. Fibroblasts obtained from

TABLE I.

Action of Shaken Chicken Serum on Homologous Fibroblasts and Sheep Erythrocytes.

Experiment No.	Serum No.	Time shaken.	Culture No.	Rate of growth of fibroblasts.		Hemolysis of sheep corpuscles.		
				Normal serum.	Shaken serum.	Concentration of serum.	Amount of hemolysis.	
							Normal serum.	Shaken serum.
		hrs.				*per cent*	*per cent*	*per cent*
1	1406	1	1445	2.00	1.50	20	10	5
		1	1445	2.00	1.00	50	50	18
		1	1445	4.00	2.00			
		1	1445	3.00	1.50	70	90	35
2	1429	7	24967	3.48	2.97			
		7	24967	2.40	1.67			
		7	24967	3.59	2.50			
		7	25071	2.80	2.70			
		7	25071	3.54	2.89			
		7	25071	3.82	1.69			
3	1737	4	1741	4.76	1.39			
		4	1741	3.47	0.83			
		4	28374	3.36	2.62			
		4	28374	3.28	2.11			
		4	28374	3.21	2.72			
		4	28374	2.50	1.76			
4	1737	8	1750	1.60	0.01	10	45	0
		8	1750	2.26	0.01	20	100	0
5	1746	4	1751	3.19	1.82	20	80	70
		4	1751	3.90	1.01			
		4	1751	5.50	5.30	30	100	100
6	1758	4	1761	3.00	2.00			
		4	1761	2.00	1.50			
7	1758	7	1798	5.41	4.50	10	10	5
		7	1798	3.59	1.00	20	40	15
		7	1798	0	0	40	100	35

pure cultures of a 10 year old strain were used for measuring the effect of chicken serum on homologous cells. The medium consisted of one volume of normal plasma, two volumes of shaken serum, and one volume of shaken serum containing 1:20 embryo juice. In the controls, the shaken serum was replaced by normal serum. The rate of growth was measured according to a technique previously described.[2]

Five sera, shaken from 1 to 8 hours, were examined (Table I). They were partly inactivated as shown by the hemolytic tests. In one case, inactivation was complete. The inhibiting action of shaken serum on fibroblasts was always found to be at least 30 per cent greater than that of unshaken serum. In the experiment in which serum was completely inactivated, the differences in the action of both shaken and unshaken sera were still more marked (Table I, Experiment 4). It was evident that partial or complete inactivation by shaking had a marked restraining effect on the activity of homologous fibroblasts.

II.

Action of Shaken Serum on Foreign Erythrocytes and on Foreign Fibroblasts.

Fresh dog serum, shaken in the same manner as chicken serum, became slightly opaque and partly inactivated (Table II). Its in-

TABLE II.

Action of Shaken Dog Serum on Chicken Fibroblasts and Sheep Erythrocytes.

Experiment No.	Serum No.	Time shaken.	Culture No.	Rate of growth of fibroblasts.		Hemolysis of sheep corpuscles.		
				Normal serum.	Shaken serum.	Concentration of serum.	Amount of hemolysis.	
							Normal serum.	Shaken serum.
		hrs.				*per cent*	*per cent*	*per cent*
1	1396	3	1402	3.5	4.5	10	100	25
		3	1402	2.5	4.0	20	85	7
2	1300	4	1423	2.0	8.0			
		4	1423	3.0	7.0			
3	1428	7	1434	2.0	3.5			
		7	1434	2.0	3.0			

[2] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

hibiting action was tested upon chicken fibroblasts taken from stock cultures of a 10 year old strain, and found very much decreased. The average width of the ring of new tissue was about twice as large in shaken as in unshaken serum. It appeared that dog serum partly inactivated by shaking became a much better medium for chicken fibroblasts.

III.

DISCUSSION AND CONCLUSIONS.

The preceding experiments showed that partial or complete inactivation of serum by shaking brought about a marked decrease in the activity of homologous fibroblasts. Generally, serum was not completely inactivated by shaking. In only one instance was the hemolytic effect of chicken serum on sheep corpuscles entirely lost after the serum had been shaken for 8 hours. In all other cases, the normal hemolytic power of chicken and dog sera for sheep erythrocytes was merely decreased. The effect of shaking varied according to certain conditions of the serum. It may be compared to the influence of heat, which differs widely according to individual sera, even when they are obtained from animals which are apparently in identical condition. Shaken serum always inhibited the activity of homologous fibroblasts more than normal serum. When chicken serum was completely inactivated by shaking, its restraining action on chicken fibroblasts became also more marked. On the contrary, dog serum partly inactivated by shaking was much less toxic for chicken fibroblasts than normal serum. Thus, shaking brought about a change in the condition of serum, against which homologous and heterologous fibroblasts reacted in an opposite manner. At the same time, the normal lytic action of serum on foreign erythrocytes decreased.

This last phenomenon is caused, as is well known, by the partial or complete destruction of alexin. The decrease of the restraining effect of shaken serum on foreign fibroblasts may be attributed to the same cause. The increase of the inhibiting action of shaken serum on homologous fibroblasts is due possibly to the disappearance of a substance favoring the activity of homologous cells. A similar hypothesis was advanced for explaining the increase of the growth-

inhibiting action of serum under the influence of senescence[3] and of heat.[1] The restraining power of adult serum on cell multiplication may be due to the antagonistic action of growth-promoting and growth-inhibiting substances, the growth-promoting substance being as unstable as alexin and certain tissue juices which have the property of increasing the rate of cell proliferation. Alexin and growth-activating substances contained in embryonic and gland tissue juices have in common the property of being destroyed by heat and by shaking. Leucocytes added to serum under certain conditions increase its hemolytic power on foreign erythrocytes and decrease its inhibiting action on homologous fibroblasts. Variations in the alexinic activity of serum under other influences are followed also by a change in its action on homologous cells. Long ago, Gengou[4] found that serum from plasma is less bactericidal than serum from blood. We observed that the lytic effect on sheep erythrocytes of dog and chicken serum from blood was sometimes more marked than that of serum from plasma, but there was often no difference in the action of both sera. At the same time, the growth of homologous fibroblasts was always more extensive in the serum from blood. The opposite effect was observed when heterologous fibroblasts were used. The growth-inhibiting power of serum from blood was still less marked on leucocytes than on fibroblasts. But embryonic juice, which greatly enhances the rate of multiplication of homologous cells, did not increase the lytic action of serum on foreign erythrocytes.

It may be concluded that, under the conditions of the experiments:

1. Chicken serum partly or completely inactivated by shaking becomes more inhibiting for chicken fibroblasts.

2. Dog serum partly inactivated by shaking becomes less inhibiting for chicken fibroblasts.

[3] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599.

[4] Gengou, O., *Ann. Inst. Pasteur*, 1901, xv, 232.

THE HYDROGEN ION CONCENTRATION OF JOINT EXUDATES IN RHEUMATIC FEVER AND OTHER FORMS OF ARTHRITIS.

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(Received for publication, May 19, 1922.)

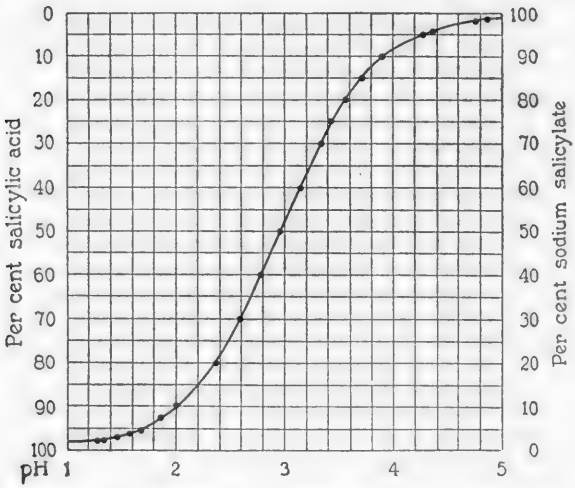
In connection with a study of acute rheumatic fever we have determined the hydrogen ion concentration of exudates aspirated from the inflamed joints of patients ill with this disease and also of exudates of patients with certain other forms of arthritis. This was done (1) to compare the reactions of the exudates in these arthritic diseases, and (2) to determine whether an acidity occurs in the inflamed joints in acute rheumatic fever sufficient to permit the liberation of free salicylic acid following salicylate therapy.

One explanation of the action of salicylates in patients with rheumatic fever has been based upon the hypothesis that free salicylic acid is liberated in the inflamed joint. If the inflammation in the joint results from the local irritation of bacteria, a bactericidal action of free salicylic acid might explain the improvement that ordinarily follows the administration of this drug. This theory originated with Binz (1), who realized that free salicylic acid could not exist in normal blood and tissues, and also that the salts of salicylates are not bactericidal in weak solutions. In subjecting sodium salicylate solutions to high CO_2 tensions *in vitro*, he found that sufficient acid was liberated to be bactericidal. The reason for the liberation of free salicylic acid in Binz' experiments is obvious: the increased CO_2 tension caused sufficient acidity to allow dissociation of the acid. He thought that a similar increased CO_2 tension occurred in the inflamed tissues in acute rheumatic fever because Ewald (2) had previously shown that such an increase could occur in certain types of inflammatory exudates. Although Binz' theory seemed unsatisfactory, it has never been conclusively disproven and is quoted in most pharmacologies.

Hanzlik and his collaborators (3), in an extensive investigation of the pharmacology of salicylates, examined joint fluids directly by the ferric alum test for the presence of free salicylic acid; the patients with rheumatic fever received full therapeutic doses of the drug. These authors found no free salicylic acid present. A criticism of this work was made by the authors themselves who state that: "Objection might be raised to the method used because of the possibility that

some CO_2 is lost when the fluid is exposed to the atmosphere, but this is to a considerable extent prevented by the presence of 'buffer' or protective substances."

Reaction at Which Free Salicylate Acid Occurs.—Hanzlik (4) also found that salicylic acid was released from mixtures of sodium salicylate and buffer salts only when the acidity was greater than pH 6.7. The iron test for salicylic acid was found to be faintly positive at a pH of 6.7 and progressively stronger with an increase in acidity from pH 6.5 to 1.0; on the alkaline side of pH 6.7 no salicylic acid was liberated. Mixtures containing 25 per cent serum



TEXT-FIG. 1. Salicylic acid-sodium salicylate curve at 40°C.

or plasma did not show any free salicylic acid between pH 7.4 and 5.9; tests were not carried out at a higher acidity. He states that: "It is conceivable that very low degrees of acidity, *i.e.*, in the neighborhood of pH = 6.8 or 6.9, might occur in closed cavities with sluggish circulation as in the articulations," but "fluids of joints and similarly enclosed regions would need to be more highly acid and freer from protein and other constituents than is probably the case in order to contain free salicylic acid and explain the therapeutic relief from salicylate medication according to the antiseptic theory."

The dissociation curve for mixtures of salicylic acid and sodium salicylate is given in Text-fig. 1. It is evident that at reactions more

alkaline than pH 5.0 the free acid constitutes less than 1 per cent or the total salicylate. Since it has been estimated that following full therapeutic doses of salicylate the concentration in the blood or joint fluid is about 0.02 per cent (3), the amount of free acid present at a pH of 6.0 would be less than 0.0002 per cent; *i.e.*, an amount that could exert no bactericidal action. This conclusion is in agreement with Hanzlik's experimental findings.

EXPERIMENTAL.

The reactions of twenty-six joint exudates have been determined. The majority of these were from patients ill with acute rheumatic fever; a number were from patients having arthritis of unknown origin (chronic arthritis, intermittent hydrarthrosis); two were from patients with definite bacterial arthritis; and one, a simple effusion, was from a patient with myocardial insufficiency and generalized edema.

In each instance the joint was aspirated with a tightly fitting Luer syringe containing a small amount of sterile paraffin oil to prevent the admission of any air bubbles. During the entire determination the fluid was prevented from coming in contact with the air.

Owing to the fact that a considerable quantity of fluid was necessary for electrometric pH determinations and that in each instance several additional cubic centimeters were needed for culture, colorimetric determinations were made in the majority of instances. When large quantities of fluid were available, both colorimetric and electrometric determinations were made, in order to obtain the factor necessary to convert colorimetric readings at room temperature to electrometric at body temperature (38°C.).

Electrometric Measurements.[1]—The electrometric determinations were made at 38°C. in the Clark cell, in a hydrogen atmosphere containing CO_2 at the tension existing in the joint fluid. The determinations were corrected for partial pressures of CO_2 and H_2O to one atmosphere of dry H_2. The solution used in the standardization of the hydrogen electrode was 0.1 N HCl whose pH at 38° was assumed to be 1.09. This brings the pH determinations to the basis of Sören-

[1] A number of these determinations were made by Dr. A. B. Hastings.

sen's standards. A detailed description of the technique employed has been recently published by Cullen (5).

Colorimetric Measurements.—The method was that described by Cullen (5) for the colorimetric determination of the hydrogen ion concentration of blood. 1 cc. of the aspirated joint fluid (usually within 4 minutes of withdrawal and before coagulation occurred) was added to a standard tube containing 20 cc. of 0.9 per cent sodium chloride solution and 7 drops of indicator; the addition was made under paraffin oil to prevent the escape of any CO_2. 1 cc. of the same joint fluid was added to another tube containing 20 cc. of saline solution without any indicator to serve as a turbidity control in the comparator. The pH determination was then made by placing the tube in a comparator block and comparing it with standard color tubes. This was done at room temperature. Phenol red was used as indicator for determinations above pH 6.8 and brom-cresol purple for those below pH 6.8. All glassware, mineral oil, and salt solution were previously tested for neutrality. As standards, Sörensen's phosphate standards were prepared in steps of pH 0.2 from pH 5.6 to 6.8 and in steps of pH 0.05 from pH of 6.8 to 8.0. To those above pH 6.8, 5 drops of 0.03 per cent phenol red solution were added to each 15 cc. of standard; to those below pH 6.8 a similar quantity of brom-cresol purple was added.

The colorimetric determination at room temperature was corrected to 38°C. by the following formula:

$$pH_{38^\circ} = \text{colorimetric pH} + 0.01\ (t - 20^\circ)\ \text{pH} - 0.21\ \text{pH}$$

in which t = room temperature and 0.21 pH represents the empirical correction for temperature ($38^\circ - 20^\circ$), dilution, and protein errors. This correction represents the average of the differences between electrometric determinations at 38°C. undiluted, and the colorimetric determinations, diluted, at 20°C.; it corresponds closely with the correction similarly found for correcting colorimetric determinations of human blood. The room temperature in these experiments varied within $\pm$ 3°C. from 20°C. and a temperature change of 0.01 pH was made for each degree of variation.

Physical Characteristics of the Exudates Examined.—The fluids aspirated from the inflamed joints of patients ill with acute rheumatic

fever were never frankly purulent. They were viscous, slightly to distinctly turbid, and usually of a pale yellowish green color. Sometimes when the exudate was aspirated from a joint during the early stage of the arthritis, the greenish yellow shade was absent and the fluid had a grayish turbidity; this effect was probably due to a higher leucocyte content. The exudates contained considerable fibrin and formed soft clots on standing. Bacteriologically, they were sterile by ordinary culture methods. The fluids from the patients with arthritis of undetermined origin were indistinguishable from some of these exudates. The joint fluid from the patient with myocardial insufficiency was less turbid, did not clot so quickly, and had a lower leucocyte count than most of the rheumatic fever exudates. The exudates of the two patients with bacterial arthritis were easily distinguished as coming from infected joints. They were purulent fluids and contained bacteria.

Results.

Hydrogen Ion Concentration.—The results are shown in Table I. With the exception of the joint exudates of the two patients with bacterial arthritis, the reactions were all slightly alkaline and approximated the normal reaction of blood. The hydrogen ion determinations of sixteen fluids from patients with acute rheumatic fever varied between pH 7.27 and 7.42. Seven fluids were examined from three patients with arthritis of undetermined origin; one of these was diagnosed as intermittent hydrarthrosis, one as chronic arthritis, and the third was probably arthritis accompanying serum disease. Their reactions were approximately the same as those in acute rheumatic fever, varying between pH 7.33 and 7.47. The joint fluids of the two patients with bacterial arthritis were both definitely acid; the exudate from the knee joint infected with *Staphylococcus aureus* was pH 6.69; from the one with *Streptococcus hæmolyticus*, pH 6.19. The electrometric determination of this latter fluid cannot be considered accurate as the fluid was kept over night in the ice box and heated to 56°C. for 1 hour to kill the organisms; the result, however, is sufficiently accurate to show the fluid to be definitely acid. The simple effusion from the patient with myocardial insufficiency accompanied by general edema was pH 7.34.

TABLE I.

Hydrogen Ion Concentration of Joint Exudates.

Case No.	Patient's temperature at time of aspiration.	Joint.	Length of time joint aspirated was involved.	Degree of inflammation.*	Bacteriological examination.	Determinations.			
						Colorimetric at 20°C.	Electrometric at 38°C.	Difference between colorimetric at 20°C. and electrometric at 38°C.	Colorimetric calculated at 38°C.†
Acute rheumatic fever.									
	°F.		*days*			*pH*	*pH*	*pH*	*pH*
4444	104.5	Right knee.	3	+++	No growth.	7.65	7.39	0.26	7.39
4350	102	Left wrist.	5	+	" "	7.53‡			7.32
4453	102	Right knee.	1	+++	" "	7.58	7.34	0.24	7.34
	99	Left "	7	+	" "	7.58			7.37
4367	100.5	Right "	10	+	" "	7.63‡			7.42
	104	Left "	2	++	" "	7.56‡			7.35
	104	Right "	2	++	" "	7.57‡			7.36
4417	103	" "	8	+	" "	7.54‡			7.33
	103	Left "	3	++	" "	7.54‡			7.33
	102.5	" "	5	+	" "	7.55‡			7.34
	102.5	Right "	12	+	" "	7.57‡			7.36
4473	103	Left "	1	+++	" "	7.49	7.27	0.22	7.27
	103	" "	2	++	" "	7.60			7.39
4485	103.8	Right "	1	+++	" "	7.54			7.33
	103.8	Left "	3	++	" "	7.56			7.35
4493	103	" "	4	+++	" "	7.51	7.34	0.17	7.34

* + indicates swelling only; ++, moderate inflammation; +++, marked inflammation.

† Average correction of pH 0.21 used except where actual difference is given.

‡ Room temperature not recorded. Variation not more than ± 3°C. from 20°C.

TABLE I—*Concluded.*

Case No.	Patient's temperature at time of aspiration.	Joint.	Length of time joint aspirated was involved.	Degree of inflammation.*	Bacteriological examination.	Determinations. Colorimetric at 20°C.	Electrometric at 38°C.	Difference between colorimetric at 20°C. and electrometric at 38°C.	Colorimetric calculated at 38°C.†
Arthritis of undetermined origin (includes chronic arthritis and intermittent hydrarthrosis).									
	°F.		*days*			*pH*	*pH*	*pH*	*pH*
4451	101	Right knee.	2	+++	No growth.	7.54			7.33
	102	Left "	5	+++	" "	7.65			7.44
4428	99	Right "	?	+	" "	7.61	7.43	0.18	7.43
	100	" "	2	+	" "	7.67	7.42	0.25	7.42
	99	" "	2	+	" "	7.62	7.42	0.20	7.42
	98.5	" "	30 *mos.*	+	" "	7.68			7.47
4449	99	Left "	5	+	" "	7.60	7.42	0.18	7.42
Bacterial arthritis.									
			days						
4262	104	Right knee.	8	+++	*Staphylococcus aureus.*	6.90			6.69
4467	104.2	Left "	?	+++	*Streptococcus hæmolyticus.*	6.40§ 6.10‖	5.89	0.21	6.19
Joint effusion, myocardial insufficiency.									
4458	102	Left knee.	?	+	No growth.	7.55			7.34

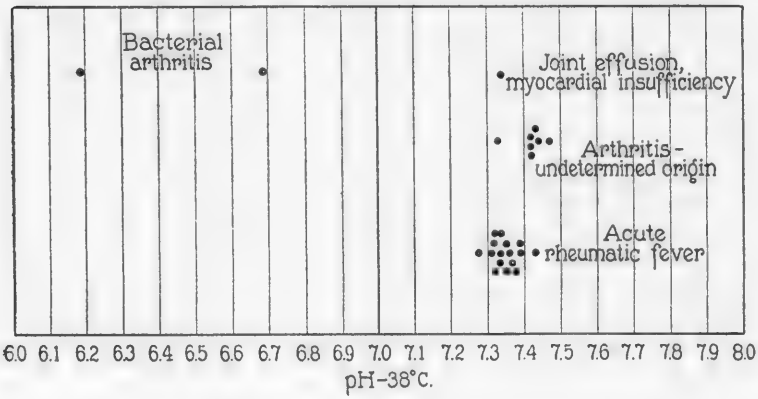
§ Within 4 minutes of aspiration.

‖ Determined after standing in ice box over night and heated at 56°C. for 1 hour to kill bacteria.

DISCUSSION.

A comparison of the results is indicated in Text-fig. 2. It shows that the joint exudates on the basis of their reactions fall into two groups; the one having a slightly acid, the other a slightly alkaline reaction. The fluids containing bacteria fell into the first, the sterile fluids into the second. In general, these findings correspond with those of other investigators who have recently made hydrogen ion

determinations on various types of exudates. Shearer and Parsons (6) found the purulent spinal fluid of epidemic cerebrospinal meningitis to be about pH 6.9. Lord (7) found the pH of pneumonic lung exudates to be as low as 5.4. Shade and his coworkers (8) determined the reactions of many exudates electrometrically; pus from acute infections varied in pH between 5.96 and 6.57; pus from chronic inflammations such as tuberculosis between 6.58 and 7.00; serous exudates as in tuberculous pleurisy between 7.00 and 7.09; and non-inflammatory transudates between pH 7.17 and 7.24.


Bacterial arthritis
Joint effusion, myocardial insufficiency
Arthritis - undetermined origin
Acute rheumatic fever
6.0 6.1 6.2 6.3 6.4 6.5 6.6 6.7 6.8 6.9 7.0 7.1 7.2 7.3 7.4 7.5 7.6 7.7 7.8 7.9 8.0
pH-38°C.

TEXT-FIG. 2. Hydrogen ion concentration of joint exudates.

It seems to us that only in the instance of purulent exudates would the liberation of free salicylic acid be at all possible. If acid is liberated in these exudates it is not sufficient to be bactericidal, as clinical observations have never shown any improvement in purulent arthritis following salicylate treatment. As Hanzlik has previously concluded from direct examinations that free salicylic acid could not be demonstrated in the joint exudates of patients with acute rheumatic fever, and our findings indicate that free acid could not possibly exist, it is evident that the local antiphlogistic effect of the drug is due to some other factor than free salicylic acid.

The determination of the reactions of these fluids has thrown some light upon the nature of the pathological process in the arthritis of

acute rheumatic fever. The results of various workers already mentioned show that when bacteria such as hemolytic streptococci, staphylococci, pneumococci, meningococci, or tubercle bacilli are present in an exudate in sufficient numbers to be detected, either by cultural or microscopic examination, the reaction of that exudate is always more acid than blood. We have evidence to show that the exudates in experimental arthritis of animals inoculated with green streptococci are also acid. From these results it would seem that if green streptococci were growing in the joint fluids of rheumatic fever patients, one should expect those fluids to be acid. But our examinations have shown that this is not the case. It is highly improbable, therefore, that one could cultivate streptococci from such exudates. This is in agreement with our bacteriological studies.

Another possibility must be considered: the etiologic agent—whatever it may be—may exist only in the capsule or contiguous tissues; and the exudate may be similar in nature to the serofibrinous fluid often found in pleurisy secondary to pneumonia. In many instances these pleuritic fluids are sterile and the patients recover without developing empyema. While there are not sufficient data concerning the reaction of such fluids, from the report of Shade we would expect them to be more alkaline than pH 7.0. Further observations should be made concerning the reaction of sterile fluids in serous cavities contiguous to foci of inflammation resulting from bacterial infection. When this information is available we shall be in a better position to evaluate properly the relation of our findings to the etiology and pathology of rheumatic fever.

The slight variation in the reactions of the exudates of acute rheumatic fever was not dependent upon the severity of the inflammation as evidenced clinically. The exudates from acutely inflamed joints were of about the same pH as those from joints in which the clinical signs of acuteness had disappeared and the only remaining evidence of involvement was the presence of fluid. The most acid exudate, having a pH 7.27, was found in a highly inflamed joint; the most alkaline fluid, having a pH 7.42, was found in a joint from which all acute signs of inflammation had disappeared; one highly inflamed joint, however, had a pH of 7.39 (Table II).

TABLE II.

Acute Rheumatic Fever.

Comparison of Exudates from Acutely Inflamed Joints with Those in Which Signs of Acute Inflammation Had Subsided.

Exudates from joints acutely inflamed	$pH_{38°} = 7.27-7.39$
" " " in which inflammation had subsided	$pH_{38°} = 7.31-7.42$

SUMMARY AND CONCLUSIONS.

1. The hydrogen ion concentration of joint exudates aspirated from patients ill with acute rheumatic fever, arthritis of undetermined origin, and bacterial arthritis was determined. The hydrogen ion concentrations of the joint exudates from patients with acute rheumatic fever approximated the normal reaction of blood, varying from pH 7.27 to 7.42. Exudates from patients with arthritis of undetermined origin varied in pH from 7.33 to 7.47. The pH of a joint effusion occurring in a patient with myocardial insufficiency was 7.34. Bacteriologically, all of these fluids were sterile by ordinary means of cultivation. An exudate aspirated from a knee infected with *Staphylococcus aureus* had a pH of 6.69, while that from a patient having an arthritis due to *Streptococcus hæmolyticus* was also acid, having a pH of 6.19.

2. Since a definitely acid medium is necessary for the liberation of free salicylic acid and since all of the joint fluids from patients with acute rheumatic fever were slightly alkaline, no free salicylic acid could possibly exist in such joint fluids following the administration of salicylates.

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ON ALIMENTARY LEUCOCYTOSIS IN ITS RELATION TO THE "CRISE HÉMOCLASIQUE" OF WIDAL.

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During the last few years Widal and his coworkers have shown that under certain conditions a characteristic complex of changes occurs in the blood, a complex denoted by Widal as "*crise hémoclasique*," or "*crise colloïdoclasique*."

If a normal animal is injected intravenously with a certain dose of peptone the blood pressure will fall, the clotting time of the blood will be decreased, the number of leucocytes in the peripheral blood stream will decrease, and the refractive index of the serum will change. Widal (1) showed that in the normal dog after a protein meal higher cleavage products of protein, *i.e.* peptones or albumoses, are absorbed from the intestine, and the occurrence of these cleavage products in the portal blood stream can be demonstrated by physiological means. In normal animals these cleavage products cannot enter the general blood stream, since they are fixed in the liver ("*pouvoir protéopexique*" of the liver). If, however, the liver cells are damaged, they lose part of their fixing power, and albumoses or peptones will escape in the general blood stream and will produce changes in clotting time, in refractive index, in leucocyte count, and so forth, and thus a *crise hémoclasique* will ensue. Such a *crise hémoclasique* can also be produced in other ways; *viz.*, by injection of a specific protein into an individual sensitized to that protein (anaphylactic shock), by injections of certain colloids or of drugs, such as salvarsan, to predisposed individuals, etc. The main point of interest, with regard to the work published in the present paper, is the observation of Widal and his coworkers (2) that in cases of hypersensitiveness to certain foodstuffs in men the ingestion of these foodstuffs will produce a *crise colloïdoclasique* even in the absence of any demonstrable damage of

liver cells. The interrelation of symptoms of hypersensitiveness and of those of a *crise hémoclasique* is, according to Widal, so close that in doubtful cases the finding of a *crise hémoclasique* after ingestion of a certain foodstuff will give an indication as to the nature of the causative agent of the symptoms of hypersensitiveness (migraine, asthma, hay fever, etc.) and thus clear up the diagnosis. Since, according to Widal, the most prominent feature of the *crise hémoclasique* is the decrease in leucocyte count within 20 to 40 minutes after ingestion of the foodstuff or drug, in many cases the diagnosis of hypersensitiveness can be made from a series of leucocyte counts at intervals of 20 minutes before and after the ingestion of a certain foodstuff.

Two of the present authors have been interested in the study of the causes and treatment of bronchial asthma. They found that in many cases of bronchial asthma and hay fever there seems to be a relation between hypersensitiveness to tuberculin and hypersensitiveness to the known or unknown causative agent of the acute attacks of asthma, and were able to prove that in many of these cases a cure or a considerable relief of the asthmatic symptoms could be obtained by desensitizing the patients to tuberculin by means of repeated injections of small doses of this (3). In the course of this work, however, they obtained the impression that in some of the asthma patients who were resistant to the tuberculin therapy the cause of the attacks of asthma was to be looked for in the intestine, and later it appeared that in a number of other cases who were benefited by tuberculin treatment an intestinal factor also played an important part. In accordance with the current opinion the outbreak of attacks of asthma in these cases was ascribed to the ingestion of one definite protein (varying from case to case, but being specific for each patient), to which the patient in question was hypersensitive. It may be stated at once that this opinion proved to be incorrect. Observations and deductions on this point will be discussed in another paper. They only wish to emphasize here that they had at that time to face the following problems:

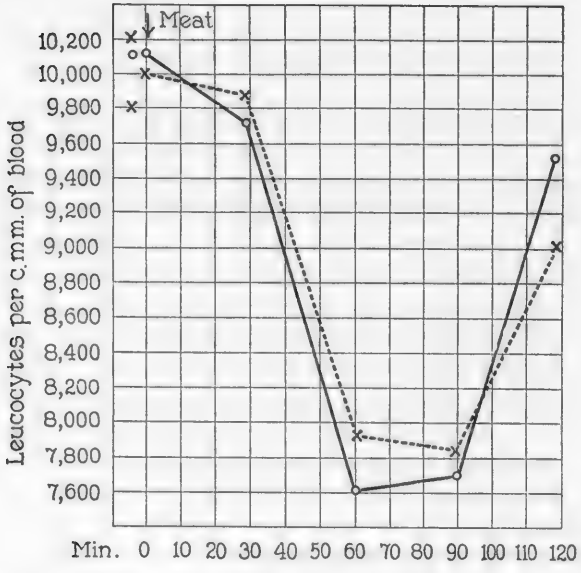
Taken for granted that certain symptoms of hypersensitiveness (attacks of asthma) in an individual are caused by the ingestion of a definite, but unknown, protein, how was this protein to be identified? Obviously the most exact way would be to feed the patient for a couple

of days on one protein only, changing after some days to another protein and going on in this way till the protein causing the attacks of asthma was found. This procedure, apart from offering considerable technical difficulties, would take a long time and, moreover, would suffer from the psychic factors which are of great influence in many cases of asthma.

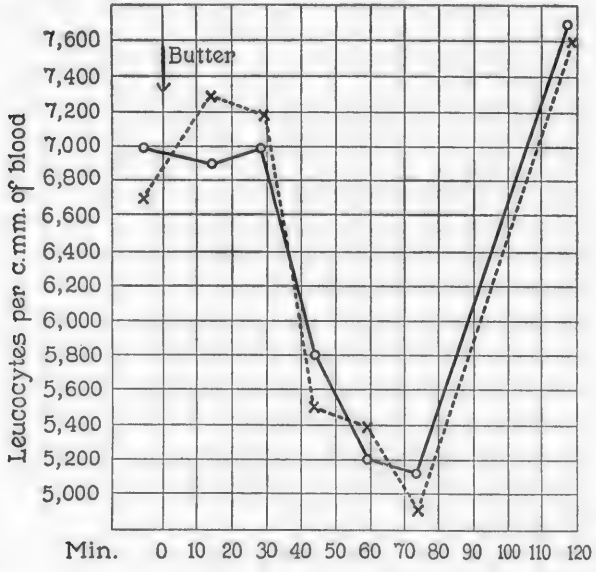
After having studied the work of Widal and his coworkers it seemed possible that the simple method indicated by him for the determination of the occurrence of a *crise colloïdoclasique*, *i.e.* counting of leucocytes in the peripheral blood stream, might be adopted for our cases. Consequently a study was undertaken of the influence of ingestion of various proteins on the leucocyte counts in a number of asthma patients. After the first investigations it proved to be necessary to include a study of other foodstuffs, *viz.* fat and carbohydrates, and to extend our investigations to the behavior of normal men. The result of this work which includes 150 observations on 50 individuals is given below.

In our first tests we followed exactly the instructions given by Widal. Leucocyte counts were made in the morning before any food had been taken, then the patient took the food to be tested, and during the next 2 hours counts were made at intervals of 20 minutes. At first three kinds of test meals were used; *viz.*, 300 cc. of milk, or three eggs, or 150 gm. of meat. Since, however, clinical observations had shown that in some of our asthma patients attacks of asthma were produced also by the ingestion of non-protein food, the investigation was extended to butter (100 gm.) and rice (150 gm.).

About the technique employed only a few words need be said. Blood was taken from the finger-tip and counts were made in a Bürker counting chamber (so that we used about the same method as Widal). In all observations to be reported here double counts were made; that is to say, either one investigator took blood in two pipettes and used two counting chambers and afterwards plotted the results obtained in each counting chamber separately, or blood was taken in two pipettes by two investigators each of whom made counts in one chamber. We wish to emphasize that the curves obtained in this way run almost entirely parallel; the technical error is certainly not higher than 5 per cent (Text-figs. 1 and 2), whereas those changes



TEXT-FIG. 1. Leucocytic counts made at the same time by two different observers. The arrow indicates the ingestion of 150 gm. of meat.



TEXT-FIG. 2. Leucocytic counts made in one experiment by one observer working with two pipettes and two counting chambers. The arrow indicates the ingestion of 100 gm. of butter.

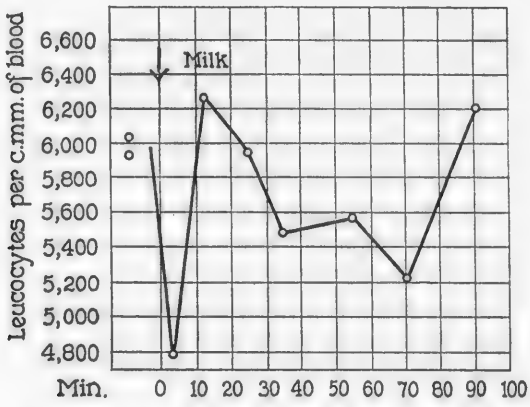
in leucocyte counts which are to be considered of clinical significance are about 20 per cent and higher, so that the method used, although not absolutely accurate, was sufficient for our purpose.

In nearly every case of asthma we found a *crise hémoclasique* after ingestion of some test meal but every case gave different results. Some reacted to milk only, others to meat, or butter, eggs, or rice only, still others reacted to butter and rice or to meat and eggs; in fact nearly every possible combination was found. Text-fig. 1 gives an instance of a definite fall in leucocytic counts after ingestion of meat, Text-fig. 2 after butter in another patient.

The fact that a fall in leucocytic count was found after ingestion of butter as well as after ingestion of protein showed that Widal's explanation as to the origin of the *crise* certainly could not hold good in our cases, since it is not very likely that ingestion of butter would cause after 20 minutes an increase in the albumose content of the portal blood stream. It is fair to state here that we are not the first to show that a fall in the number of leucocytes in the peripheral blood may occur after the ingestion of non-protein matter, as Widal has already found the same phenomenon after the ingestion of small amounts of glucose in diabetics (1).

Apart from this consideration, another observation was made which opened the question whether a leucopenia found in many of the cases in which the test meal consisted of protein could be explained on the same basis as in Widal's cases. Thus a considerable fall in the number of white blood cells (to more than 20 per cent below the original number) ensued within much less than 20 minutes after the ingestion of the food, so that it seemed hardly possible that in so short a time protein matter would be decomposed to albumoses and absorbed so as to produce a *crise*. This led us to decrease gradually the interval between the ingestion of the food and the first counting following it, so that in the end this interval was only 2 minutes. It was surprising to find that even in this last case a fall in the number of leucocytes occurred in some individuals. Text-fig. 3 gives an instance of such an incident. Before the ingestion of food the number of leucocytes was 6,000 per c.mm. The patient drank 200 cc. of milk, and 2 minutes afterwards blood was taken from a finger-tip; the number of white cells fell to 4,800; *i.e.*, a fall of about 20 per cent.

The end of this curve is equally interesting; it shows that 10 minutes after the ingestion of milk the number of leucocytes was about normal again; 10 minutes later, *i.e.* 20 minutes after the taking of milk, the curve shows a tendency to go down again, and gradually the number of leucocytes decreases and seems to reach a (second) minimum about 70 minutes after the drinking of the milk. Text-fig. 4 gives a similar curve after a test meal of rice. The original count of white cells was 9,000 in this case; 2 minutes after the eating of rice the leucocytes fell to 6,800, *i.e.* a drop of about 25 per cent; during the next three counts, made at intervals of 3 minutes, they increased to about 8,200,

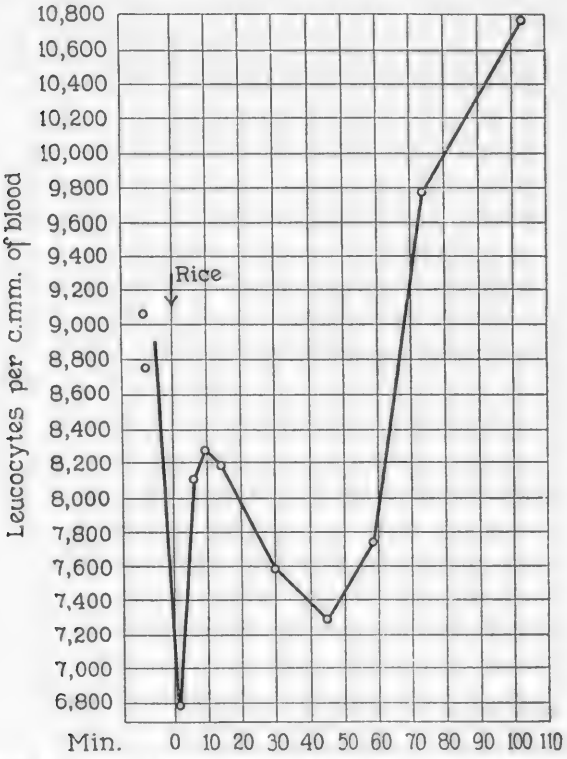


TEXT-FIG. 3. Decrease in the number of leucocytes within 2 minutes after the ingestion of milk. The arrow indicates the ingestion of 200 cc. of milk.

then followed a second drop, and subsequently a rise, far above the original number. This rise in leucocytes, which often occurs if counts are made during a sufficiently long time, is nothing else than the so called physiological leucocytosis after ingestion of food and will be left out of consideration, since the phenomena which are of interest here all occur before this physiological leucocytosis sets in.

The fact that a fall in number of white cells often occurs within 2 minutes after the ingestion of food excludes any possibility that *this* fall has a connection with absorption of cleavage products of the food administered; very likely it is caused by some reflex action from the alimentary canal, and we consider it highly probable that the phenom-

enon is only an expression of a change in the distribution of the white cells in the different regions of the blood stream. This opinion is strengthened by two observations; first, it was found that during this initial fall no characteristic change in the leucocyte formula



TEXT-FIG. 4. Decrease in the number of leucocytes within 2 minutes after the ingestion of rice. The arrow indicates the ingestion of 150 gm. of cooked rice.

occurs, and secondly, it was shown that the red cells show a decrease in number nearly parallel with that of the white cells.

The considerations mentioned made it probable that the fall in the number of leucocytes observed in the asthmatics was not to be considered as an expression of a pathological state in the individual

tested, but merely as a physiological occurrence. The correctness of this surmise was proved by finding exactly the same decrease in the number of leucocytes in normal individuals after a test meal of milk, meat, rice, and other foodstuffs. From this investigation on normal individuals it was found that in many cases a curve obtained by plotting the number of leucocytes of the peripheral blood counted at very short intervals after the ingestion of food will show the characteristics found in Text-fig. 3; *i.e.*, shortly after the test meal a sharp fall in white cells occurs. As this fall may last a few minutes or even a little longer, it is uncertain whether it will be detected if counts at intervals of 20 minutes are made. After the first fall comes a rise to about the original level, and subsequently there is a gradual fall followed by a final rise (physiological leucocytosis).

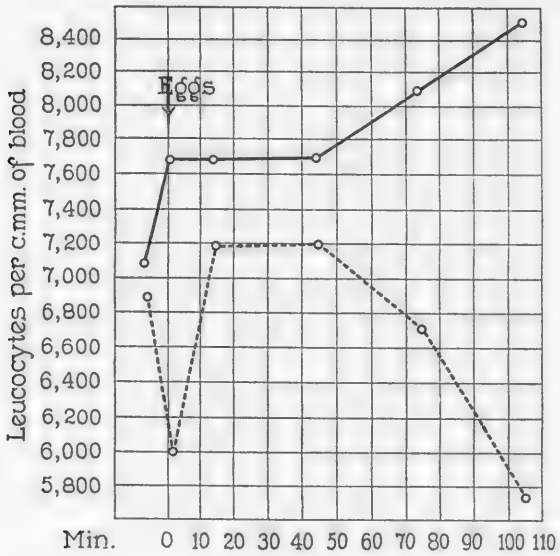
It must be stated, however, that although this type of curve frequently occurs, in other instances the curve may show a different shape. There may occur a sharp rise after some minutes, instead of a fall, or the second minimum may be absent and even replaced by a rise, and so forth.

The facts described above are in agreement with the findings of Dorlencourt and Banu (4) who studied digestive leucocytosis in normal infants. They found a decrease in leucocytes immediately after the ingestion of milk; this was followed by an increase and subsequently by a second decrease. Only after this period had passed, did the real digestive leucocytosis set in.

Adelsberger (5) and Schiff and Stransky (6) also found in normal infants and children a decrease in the number of leucocytes of short duration following shortly after the ingestion of milk and other proteins, fats, and carbohydrates.

After it was found that in many cases ingestion of food, either of protein or of non-protein nature, may cause an initial fall in the number of white cells, which may last for 5 to 20 minutes, and which may be followed first by a rise and afterwards by a second fall eventually followed by a second rise, and since it was also found that the time during which these falls and rises are demonstrable may vary from case to case, the question arose whether curves obtained by plotting the results of blood counts made at intervals of 20 or 30 minutes gave a true picture of the change in the number of leucocytes occurring in

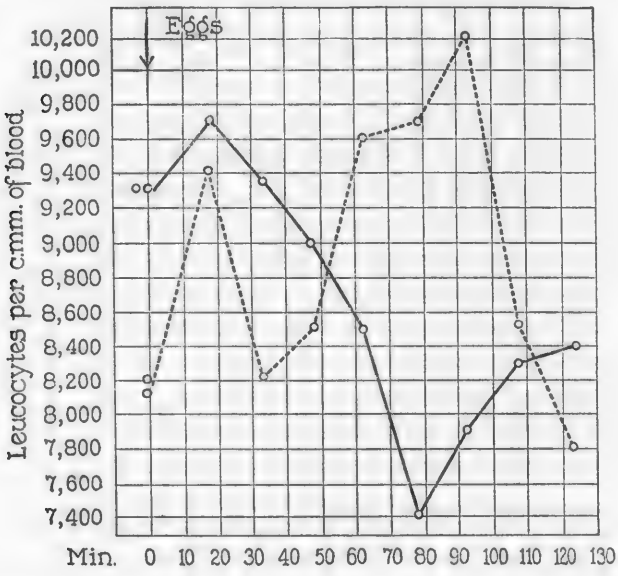
the blood. In fact it seemed very likely that results obtained in this way might be to a large degree dependent on mere chance, and the finding of a decrease of the number of white cells after the ingestion of some foodstuff could not be considered as an indication of the presence of a *crise hémoclasique* nor be an indication that this particular foodstuff was in any way related to pathological phenomena occurring in the body. To test this supposition control estimations



TEXT-FIG. 5. Leucocytic counts made by the same observer with the same food on the same patient but on 2 different days. The arrow indicates the ingestion of three eggs.

were carried out on a number of patients and normal individuals. In these cases blood counts were made at intervals of 20 minutes after the ingestion of eggs or other foodstuffs, and 2 or 3 days later the tests were repeated on the same individual, with the same foodstuffs. The curves obtained on 2 different days sometimes agreed with each other, but often also there was a considerable disagreement (Text-figs. 5 and 6). In Text-fig. 5 the first observation revealed an initial fall of leucocytes of about 13 per cent followed by a second fall of

about 20 per cent. In the second observation on the same person with the same food, only a rise in leucocytes was found. Text-fig. 6 shows a marked discrepancy between the findings on 2 different days. Attention should be called to the fact that these discrepancies cannot be ascribed to technical errors since two estimations on the same patient on the same day, even if the countings were made by different investigators, showed a nearly complete agreement (see Text-fig. 1).



TEXT-FIG. 6. The same experiments as in Text-fig. 5 but on another patient.

DISCUSSION.

Before entering into a discussion of the results we want to state that our work does not enable us to criticize Widal's conception of an interrelation of symptoms of hypersensitiveness, or anaphylactic shock, with the occurrence of a *crise hémoclasique* (*colloïdoclasique*). In all their former work and in the main part of their later work Widal and his coworkers founded their diagnosis of *crise hémoclasique* not on *one* symptom but on a complex of four or more symptoms. Later

Widal stated that since the occurrence of a fall in the number of white cells in the peripheral blood was the most prominent part of the *crise*, it was sufficient for the diagnosis of the same; we believe that our investigations have proved this last statement to be incorrect. We found a fall of white cells at various periods—from 2 to 50 minutes—after the ingestion of a meal in normal individuals and asthmatics. Moreover, a study of the blood pressure in some of these cases at short intervals showed an absence of a fall in pressure, so that there is no doubt that in our cases the fall of leucocytes in the peripheral blood was not an indication of the existence of a *crise colloïdoclasique*.

Soon after the appearance of Widal's papers on the *crise colloïdoclasique* a number of other workers started investigations as to the occurrence of *crises* in various conditions. Often the only criterion used for the diagnosis of a *crise* was the counting of the white blood cells. It is clear that as a consequence of our studies related above, those results can no longer be considered as conclusive. The fundamental observations of Widal and his coworkers, however, remain untouched by our work.

CONCLUSIONS.

Counts of the number of white blood cells at short intervals after the ingestion of a meal (meat, eggs, milk, rice, or butter) in normal individuals and in a number of asthmatics reveal the following facts.

As a rule, a sharp fall in the leucocytic curve occurs within 1 or 2 minutes after the meal; generally the curve rises within 10 to 20 minutes, but often a second fall follows 30 to 50 minutes after the meal. This may be followed by a slow rise in the curve (physiological leucocytosis). The first sharp fall is often accompanied by a similar decrease in red cells, the leucocytic formula is not changed, the blood pressure also remains unchanged, and this makes it probable that the leucopenia observed is only a manifestation of a change in distribution of the blood in different regions of the body.

Not infrequently the leucocyte curve after ingestion of food shows a form differing considerably from that described above. Counts of white cells made at intervals of 20 minutes in the same patient at different times but after ingestion of the same food show very differ-

ent leucocytic curves. Such counts do not give evidence of the existence of a *crise hémoclasique* and consequently cannot be used to identify the causative agent of cases of hypersensitiveness to foodstuffs or drugs. Whether such an identification can be obtained if instead of simply counting white cells the whole complex of symptoms originally described by Widal as characteristic for a *crise hémoclasique* is used, remains undetermined by our work.

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EXPERIMENTAL RICKETS IN RATS.

VII. The Prevention of Rickets by Sunlight, by the Rays of the Mercury Vapor Lamp, and by the Carbon Arc Lamp.

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Plate 43.

(Received for publication, May 29, 1922.)

It is now well established that infantile rickets can be prevented or cured by means of sunlight, or light from various artificial sources. Recently it has been shown by Hess, Unger, and Pappenheimer (1), and by Powers, Park, Shipley, McCollum, and Simmonds (2), that light is able to exert a similar favorable influence on the experimental rickets of rats. For the past year a large series of experiments, some of which have been reported in abstract as the work progressed, has been carried out in this laboratory with sunlight and rays of different kinds under a great variety of conditions. The investigation has included a study of the effect of variation of intensity, of transmission, or of reflection of light, of duration of exposure, of temperature, of the diet of the experimental animals, and the pigmentation of the skin, as well as other factors. A summary of the results of many of these experiments is incorporated in Tables I to VIII.

The Effect of Sunlight.

For all experiments young rats about 40 to 50 gm. in weight were used. They were kept in a darkened room at all times. After an interval of about 21 days they were radiographed for the appearance of rickets at the epiphyses of the knee joints, and were killed after a total period of 25 to 28 days. In almost every instance the bones

TABLE I.

Protective Experiments with Sunlight.

Rat No.	Diet.	Weight.		Sunlight exposure.		Rickets.		
		Initial.	Final.	Length of time daily.	Total.	Radiogram.	Pathological examination.	
							Gross.	Microscopic.
		gm.	*gm.*		*hrs.*			
246	No. 84	30	32	None (darkness).		Rickets.	Rickets.	Rickets.
247		30	30				"	"
248		20	24				"	"
436		40	50			Rickets.*		
437		44	50			" *		
438		50	60			"	Rickets.	Rickets.
249	No. 84	44	54	15 min. (24 days).	6	Negative.	Negative.	Negative.
250		36	40			"	"	"
251		30	32			"	"	"
439		42	52	15 " (14 ").	8	"	"	"
440		40	52			"	"	"
441		50	56	30 " (9 ").		"	"	"
442		44	50			"	"	"
262	No. 84 + 25 mg. of K_2HPO_4.	54	70	None (darkness).			Rickets.	Rickets.
263		52	72			Rickets.	"	"
264		44	64			Slight.	"	"
443		30	40			Rickets.*		
444		34	48			"	Slight.	Slight.
445		34	50			" *		

259	No. 84 + 25 mg. of K_2HPO_4.	40	60	15 min. (27 days).	8	Negative.	Negative.	Negative.
260		48	66			"	"	"
261		50	76			"	"	"
121	No. 84 + 75 mg. of K_2HPO_4.	40	45	None (darkness).			Negative.	Negative.
122		32	50			Negative.	"	"
123		26	44				"	"
449		30	40			Rickets.	Slight.	Very slight.
450		34	46			Slight (?).	Negative.	Negative.
451		32	40			"	"	"
124	No. 84 + 75 mg. of K_2HPO_4.	36	52	Indefinite.		Negative.	Negative.	Negative.
125		36	36			"	"	"

* Animals not killed; used for curative experiment.

were subjected to microscopic examination, and the final criterion as to the presence of rickets was the histological, rather than the radiographic picture.

Experiments with sunlight are difficult to carry out satisfactorily on account of the variability in the degree of sunshine, which makes exact quantitative work impossible. Nevertheless, as shown in Table I, daily exposures of 15 minutes sufficed to protect rats, fed a standard rickets-producing diet, which was adequate in its calcium but inadequate in its phosphorus content. This diet contained about 86 mg. of phosphorus per 100 gm. of diet; the addition of about 75 mg. of phosphorus to this diet is required to protect rats from rickets (Diet 85). The effect of sunlight may, therefore, be stated to have been equivalent to about doubling the quota of phosphorus. In considering the qualitative variability of the sun, it was noted in two series of experiments, not included in the tables, that the same degree of protection could not be obtained during November and December in rats on this diet. No doubt this is to be ascribed to the comparative lack of ultra-violet radiation furnished by the sun in the temperate zone at this season of the year, and is a factor to be considered in employing heliotherapy in infantile rickets.

As stated elsewhere, the potency of the sun is lost after its rays have traversed window glass (3). This observation is confirmed by experiments on rats. In the tests which are shown in Table II flint glass, 3.5 mm. in thickness, was employed. In another experiment window glass, 4.1 mm. in thickness, was employed with radiations from the mercury vapor lamp, as well as from the carbon arc lamp, for periods of 15 minutes. In both of these tests the animals developed marked rickets. A spectrogram of the second glass filter,[1] demonstrated that it obstructed rays shorter than 334$\mu\mu$ in length.

Another experiment was devised so that the sunlight was reflected from a smooth white surface and did not impinge directly on the rats. It will be noted (Table II) that this arrangement permitted access of a certain amount of effective radiation to the animals, an amount not adequate to protect them, but sufficient to prevent the development of the severe grade of rickets which came about either in darkness or when window glass was interposed.

[1] This was supplied by the Corning Glass Works, Corning, N. Y.

TABLE II.

Protective Experiments with Sunlight.
Direct, Transmitted, and Reflected Light.

Rat No.	Diet.	Weight.		Sunlight exposure.		Rickets.			Inorganic P per 100 cc. of blood.
		Initial.	Final.	Length of time daily.	Total.	Radiogram.	Pathological examination.		
							Gross.	Microscopic.	
		gm.	*gm.*		*hrs.*				*mg.*
528	No. 84	56	70	None (darkness).		Rickets.	Rickets.	Moderate.	5.2
530		48	60			"	"	"	4.9
535	No. 84	60	72	Direct.		Negative.	Negative.	Negative.	
536		60	68	30 min. (12 days).	20	"	"	"	
524		48	54	60 " (14 ").		"	"	"	
532	No. 84	60	70	Transmitted.		Rickets.	Rickets.	Moderate.	3.75
533		52	54	30 min. (12 days).	20	"	"	"	
534		58	60	60 " (14 ").		"	"	"	3.11
525	No. 84	40	60	Reflected.		Rickets.	Rickets.	Slight.	3.0
529		50	70	30 min. (12 days).	20	"	"	"	3.0
527		56	72	60 " (14 ").		"	"	"	2.5

Experiments with the Mercury Vapor Quartz Lamp.

The rays from the mercury vapor lamp have been used successfully in infantile rickets by Huldschinsky (4) and others. They have also been employed in experimental rickets by Powers, Park, and their associates (5), and by Hess, Unger, and Pappenheimer (3). The rays of this lamp are referred to sometimes as artificial sunlight, but differ markedly from those of the sun in that their spectrum is linear and not continuous, and they do not extend so far in the region of the infra-red but much further in the region of the ultra-violet. Table III demonstrates that they are capable of affording marked protection against rickets when given in small amount. It is not necessary to treat the animals with the rays for hours, as has been done in previous experiments. With an alternating current of 160 volts, exposures of 3 minutes or less, at a distance of 3 feet, were found quite sufficient to accomplish this result. It may be noted also that these animals failed to develop rickets in spite of the fact that the content of inorganic phosphate of their blood was far below the normal. In many instances, as reported by Gutman and Franz (6), only 4 mg. was obtained per 100 cc. of blood, whereas the normal content is frequently as high as 8 or 9 mg., when measured according to the method of Bell and Doisy. In rachitic rats which were kept in the dark and fed this diet, these investigators found the average inorganic phosphate to be 3.2 mg. per 100 cc. of blood. Kramer and Howland (7) described a greatly diminished concentration of inorganic phosphate in rats rendered rachitic by a diet deficient in phosphorus and in the organic factor contained in fats.

An attempt was made to protect rats from rickets by irradiation before they were placed on the experimental diet. To this end a series of animals was rayed for 6 minutes daily, at a distance of 3 feet, for a period of 5 days. It was found, however, that this preliminary irradiation did not delay the onset of the rachitic lesions or decrease their intensity, so that it may be inferred that this treatment did not enable the animals to store or mobilize any protective substance in their tissues.

There are at least three factors which determine the effect of light—the diet, the rate of growth, and the degree of pigmentation of the

skin. The question of diet and rate of growth will be referred to in the discussion of subsequent experiments. The importance of the relation of skin pigment can be demonstrated by a simple experiment (Table IV). If two groups of rats, one composed of white rats and the other of black rats (the melanotic form of the Norway rat), are given the minimal protective dose of light, it will be found that although diet and rate of growth have been the same, the black rats will develop rickets, whereas the white rats will show no rachitic lesions (Figs. 1 to 3). The table presents the results of radiographs and gross and microscopic examinations of two groups of rats subjected to a test of this kind. It is manifest that the protective rays were rendered comparatively inert by the integumentary pigment.[2] The applicability of this experiment to infantile rickets, especially to the well recognized susceptibility of the negro infant, is so evident as to require no further comment.

Experiments with the Carbon Arc Lamp.

A large number of experiments has been carried out with the carbon arc lamp. In view of the fact that its spectrum resembles sunlight more closely than that of the mercury vapor lamp, it seemed as if this source of light might be of value in rickets. This was found to be the case both in experimental and in infantile rickets. By means of this therapeutic agent rickets in infants can be readily cured, and the cure is accompanied by a surprisingly rapid augmentation of the inorganic phosphate of the blood (8). In a large series of young rats it was found that daily exposures of 3 minutes, at a distance of 3 feet, regularly prevented the occurrence of rickets, and that 2 minute exposures sufficed frequently under these conditions. When the animals were irradiated only every other day, slight rickets developed (Table V). Furthermore, treating with the rays at a distance of 6 feet for 1 hour afforded protection, although at a distance of 9 feet the same period of exposure proved inadequate.

[2] In one experiment (Table IV, Rats 589, 592, 593, 594, and 597) a somewhat larger amount of irradiation was given and protection followed. This shows that the pigment is not to be regarded as an absolute filter of ultra-violet rays.

TABLE III.

Protective Experiments with the Mercury Vapor Quartz Lamp.
Variations in Duration of Exposures.

Rat No.	Diet.	Weight.		Irradiation.		Rickets.			Inorganic P per 100 cc. of blood.
							Pathological examination.		
		Initial.	Final.	Exposure.	Distance.	Radiogram.	Gross.	Microscopic.	
		gm.	*gm.*	*min.*	*ft.*				*mg.*
528	No. 84	56	70	None (darkness).		Rickets.	Rickets.	Moderate.	3.07
530		48	60			"	"	"	
598		88	90			"	Marked.	"	3.0
568		36	58			"	"	"	2.45
569		38	54			"	"	"	2.0
726	No. 84	42	50	½	3	Rickets (?).	Slight.	Slight.	727, 729 2.47
727		44	50			Negative.	Very slight.	" (osteoporosis).	
728		42	44			"	" "	Minimal.	3.0
729		40	44			Slight.	Slight.	Partial protection.	
722	No. 84	40	40	1	3	Negative.	Very slight.	Slight (healing).	4.56
723		50	50			Rickets.	Slight.		
724		52	64			"	"	" "	3.95
725		50	50			"	Very slight.	"	
872	No. 84	74	80	3	3	Negative.	Negative.	Negative.	4.5
873		50	50			"	"	"	
874		70	64			"	"		4.2
875		70	54			"			

730	No. 84	44	52	15	3	Negative.	Negative.	Negative.	
731		42	50			"	"	"	
732		42	50			"	"	"	
733		44	50			"	"	"	
570	No. 84	40	54	½, 1, 1½, 2	3	Negative.	Very slight.	Negative.	3.75
571		40	56			"	" "	"	
599		90	92			" (flattened out).	Slight.	Minimal.	3.6
600	No. 84	90	95	1, 2, 3, 6	3	Negative.	Negative.	Negative.	5.35
572		36	50			"	"	"	5.42
573		40	58			"	"	"	
574	No. 84	38	50	2, 4, 6, 12	3	Negative.	Negative.	Negative.	3.8*
575		38	50			"	"	"	
601		98	100			" (flattened out).	"	"	4.34

* Test questionable.

TABLE IV.

Protective Experiments with the Mercury Vapor Quartz Lamp.
White and Black Rats.

Color of rat.	Rat No.	Diet.	Weight.		Irradiation.		Rickets.			Inorganic P per 100 cc. of blood.
			Initial.	Final.	Exposure.	Distance.	Radiogram.	Pathological examination. Gross.	Pathological examination. Microscopic.	
			gm.	*gm.*	*min.*	*ft.*				*mg.*
White.	860	No. 84	70	70	1	3	Negative.	Negative.	Negative.	
	861		58	60			"	"	"	
	862		60	70			"	"	"	
White.	857	No. 84	60	80	1½	3	Negative.	Negative.	Negative.	5.45
	858		64	70			"	"	"	4.44
	859		62	70			"	"	"	
Black.	854	No. 84	50	60	1	3	Rickets.	Marked.	Extreme.	
	855		50	60			Moderate.	"	Rickets.	
	856		60	58			"	"	"	
Black.	851	No. 84	50	50	1½	3	Moderate.	Marked.	Marked.	2.92
	852		48	54			Marked.	"	"	
	853		60	60			" (fracture).	Very marked.	"	3.00
Black.	589	No. 84	90	100	½, 1, 1½, 2	3	Negative.	Very slight.	Negative.	4.11
	592		48	70			"	" "	Very slight.	3.22
	593		50	56			"	" "	Rickets.	2.90*
Black.	594	No. 84	84	100	1, 2, 3, 6	3	Negative.	Negative.	Negative.	4.41
	597		36	48			"	"	"	

* Test questionable.

The protective effect of carbon arc light was tested also on rats fed more complex diets. Table VI shows that this source of light was effective when Diet 84 was amplified with serum albumin, egg albumin, or butter; in each instance a small amount of secondary potassium phosphate was added to compensate for the diminution of phosphorus incidental to replacing 5 or 10 per cent of the flour. Similar success was obtained with a diet amplified with dried milk. For some months experiments have been in progress with a diet containing dried milk, with the object of rendering Diet 84 more complete, and at the same time providing a food which more nearly resembles the dietary of the infant. It has been found that rickets will develop if, to a dietary composed of patent wheat flour and a simple salt mixture, about 10 per cent of dried milk (desiccated by the roller process) is added. It may be found that a diet containing 5 to 10 per cent of dried milk will be of value in investigations of experimental rickets in rats. The animals which received this more complete food were protected from rickets by irradiations of 4 minutes. Moreover, they grew exceptionally well. Whereas on Diet 84 rats increase about 10 gm. in weight in the course of the experimental period, some of the animals on this diet increased 30 gm. during the 26 days which comprised the experiment (Table VI). The factor of growth is of great importance in a consideration of measures which protect against rickets, as rapid growth tends markedly to the development of rickets. Protection afforded while the rate of growth is rapid indicates, therefore, increased potency of a therapeutic agent.

As demonstrated by McCollum, Simmonds, Shipley, and Park (9), rickets may be induced in young rats, on a diet low in phosphorus and high in calcium, or, on the contrary, on a diet low in calcium and high in phosphorus. Diet 84 is the prototype of the former diet and, as shown above, fails to induce rickets when the animals are exposed daily to a few minutes irradiation with the carbon arc lamp. Diet 85C is the prototype of the low calcium and high phosphorus diet, and regularly brings about rickets, although of somewhat different type from a histological point of view. Rats maintained on this diet were protected by light as readily as those on the low phosphate ration (Table VII), a result which suggests that light is able to compensate,

TABLE V.

Protective Experiments with the Carbon Arc Light.
Variation in Duration and Distance of Exposures.

Rat No.	Diet.	Weight.		Irradiation.		Rickets.			Inorganic P per 100 cc. of blood.
		Initial.	Final.	Exposure.	Distance.	Radiogram.	Pathological examination.		
							Gross.	Microscopic.	
		gm.	*gm.*	*min.*	*ft.*				*mg.*
698	No. 84	24	30	½, 1, 1½	3	Slight.	Rickets.	Slight.	
699		40	50			Moderate.	"	Marked.	
700		44	54			Slight.	"	"	
696	No. 84	44	58	1, 2, 3	3	Slight.	Slight.	Slight.	
697		34	50			"	"	"	
1015	No. 84	54	64	3	3	Negative.	Negative.	Negative.	
1016		30	30			"	"	"	
1017		60	70			Slight.	Very slight.	Minimal.	
980	No. 84	50	70	4	3	Negative.	Negative.	Negative.	
981		80	84			"	"	"	
982		60	62			"	"	"	
983		50	60			"	"	" (slight osteoporosis).	
900	No. 84	54	70	5	3	Negative.	Negative.	Negative.	
893		80	80			"	"	"	
906		40	44			"	"	"	
907		44	50			"	"	" (slight osteoporosis).	

892	No. 84	80	84	10	3	Negative.	Negative.	Negative.	3.2
908		50	50			"	"	"	
901		40	30			"			
909		50	44			"	Negative.	Negative (slight osteoporosis).	
803	No. 84	50	62	15	3	Negative.	Negative.	Negative (osteoporosis).	5.4
804		44	58			"	"	Negative.	
805		42	60			"	"	"	4.76
806		56	64			"	"	" (osteoporosis).	4.44
749	No. 84	30	40	60	3	Negative.	Negative.	Negative.	
750		34	42			"	"	"	
751		50	60			"	"	"	
752	No. 84	30	40	60	6	Negative.	Negative.	Negative.	
753		30	38			"	"	"	
754		54	60			" (?).	" (?).	Minimal.	
759	No. 84	22	24	60	9	Slight.	Slight.	Slight.	
760		28	38			Moderate.	"	Moderate.	
761		24	30			"	Moderate.	"	
762		20	30			Slight.	Slight.	Slight.	
1125	No. 84	30	40	5 (every other day).	3	Moderate.	Marked.	Marked.	
1126		50	56			"	Slight.	Slight.	
1127		50	54			Marked.	Marked.		
1128		50	60			"	Slight.	Slight.	

TABLE VI.

Protective Experiments with the Carbon Arc Light.
Effect of Diet.

Rat No.	Diet.	Weight.		Irradiation.		Rickets.			Inorganic P per 100 cc. of blood.
		Initial.	Final.	Exposure.	Distance.	Radiogram.	Pathological examination.		
							Gross.	Microscopic.	
		gm.	*gm.*	*min.*	*ft.*				*mg.*
1015	No. 84	54	64	3	3	Negative.	Negative.	Negative.	
1016		30	30			"	"	"	
1017		60	70			Slight.	Very slight.	Minimal.	
980	No. 84	50	70	4	3	Negative.	Negative.	Negative.	
981		80	84			"	"	"	
982		60	62			"	"	"	
983		50	60			"	"	" (slight osteoporosis).	
996	No. 84 + 10 per cent serum albumin + 50 mg. of K_2HPO_4.	60	60	4	3	Negative.	Negative	Negative (osteoporosis).	5.0
997		68	70			"	"		
998		60	68			"	"	" (moderate osteoporosis).	6.6
999		60	70			"	"	Negative (osteoporosis).	
992	No. 84 + 5 per cent butter + 25 mg. of K_2HPO_4.	54	60	4	3	Negative.	Negative.	Negative.	5.4
993		60	64			"	"	"	
994		60	54			"	"		5.0
995		70	80			"	"	Negative.	

984	No. 84 + 10 per cent egg albumin + 50 mg. of K_2HPO_4.	58	64	4	3	Negative.	Negative.	Negative (slight osteoporosis).	4.45
986		60	64			"	"	Negative.	
985		60	88			Slight.	"	"	4.8
987		40	60			"	Very slight.	Minimal (healing).	4.0
1018	7.2 per cent dry milk + 5 per cent No. 84 salt mixture* + 87.8 per cent patent wheat flour.	48	62	None.		Moderate.	Marked.	Moderate.	4.45
1019		54	74			"	Moderate.	Slight.	5.0
1020		50	70			Marked.	"	"	
1021		50	64			"	"	"	
1022	11 per cent dry milk + 5 per cent salt mixture + 84 per cent patent wheat flour.	50	70	None.		Moderate.	Moderate.	Slight.	7.15
1023		60	90			"	Marked.	"	6.66
1024		50	80			"	Moderate.	Moderate.	5.0
1025		50	70			"	"	Slight.	
1294	10 per cent dry milk + 5 per cent salt mixture + 85 per cent patent wheat flour.	64	100	4	3	Negative (?).	Very slight.	Negative.	
1295		30	60			" (?).	" "	"	
1296		38	64			" (?).	" "	"	

* Calcium lactate ..... 2.9 per cent.
Sodium chloride ..... 2.0 per cent.
Ferric citrate ..... 0.1 per cent.

TABLE VII.

Protective Experiments with the Carbon Arc Light.
High Phosphorus, Low Calcium Diet.

Rat No.	Diet.	Weight.		Irradiation.		Rickets.		
							Pathological examination.	
		Initial.	Final.	Exposure.	Distance.	Radiogram.	Gross.	Microscopic.
		gm.	*gm.*	*min.*	*ft.*			
975	No. 84	50	72	15	3	Negative.	Negative.	Negative.
976		62	67			"	"	"
977	No. 85C*	47	50	15	3		Negative.	Negative.
979		35	37				"	Almost negative.
419	No. 85C	40	36	None.		Negative (?).		Rickets (atypical).
420		46	42			" (?).		" "
421		52	50			" (?).		" "
422		54	53			" (?).		" "

* Patent flour..... 95.0 per cent.
Sodium chloride..... 2.0 per cent.
Potassium phosphate (secondary)..... 2.9 per cent.
Ferric citrate..... 0.1 per cent.

to an important degree, for a dietary deficiency of either calcium or phosphorus.

It seemed worth while to ascertain whether variations of temperature exert an effect on the protective action of light. For many years some have attributed the low incidence of infantile rickets in southern countries and in the tropics to the favorable influence of heat. Furthermore, ordinary chemical reactions are markedly accelerated by an increase of temperature. The test was planned so as to bring about a range of temperature of about 10°C. In order to accomplish this, some groups of rats were treated with the rays at room temperature, whereas others were placed in cages superimposed on ice. By this means temperatures of 29°, 23°, and 18°C. were established. The exposures were made for the standard period of 3 minutes at a distance of 3 feet. As shown in Table VIII fully as great protection was afforded at 18°C. as at 29°C., almost no rickets developing in groups of rats exposed at either temperature. The effect of lower or of higher temperatures was not tested.

CONCLUSION.

Young rats on a diet low in phosphorus can be protected from rickets by irradiations with sunlight for about 15 minutes daily. In the winter months, however, this degree of light was found insufficient. The effective rays of the sun, in the intensities studied, did not penetrate window glass. They manifested some protective value after reflection from a smooth white surface.

Irradiation of a few minutes with the rays of the mercury vapor lamp suffices to protect rats against rickets. This is true likewise of the rays from the carbon arc lamp. A standard protective dose of radiation can be formulated for rats on a standard diet.

Light is able to prevent the occurrence of rickets in rats fed a rickets-producing diet characterized either by a low phosphorus and high calcium content, or a high phosphorus and low calcium content.

Moderate variations in temperature do not alter the effective action of light rays. Pigmentation of the skin markedly lessens their effect, as demonstrated by the failure of a standard dose to protect black rats.

TABLE VIII.

Protective Experiments with the Carbon Arc Light.

Effect of Temperature.

Rat No.	Diet.	Weight.		Irradiation.		Temperature.	Rickets.			Inorganic P per 100 cc. of blood.
							Radiogram.	Pathological examination.		
		Initial.	Final.	Exposure.	Distance.			Gross.	Microscopic.	
		gm.	*gm.*	*min.*	*ft.*	°C.				*mg.*
1015	No. 84	54	64	3	3	29	Negative.	Negative.	Negative.	
1016		30	30				"	"	"	
1017		60	70				Slight.	Very slight.	Minimal.	
980	No. 84	50	70	4	3	29	Negative.	Negative.	Negative.	
981		80	84				"	"	"	
982		60	62				"	"	"	
983		50	60				"	"	" (slight osteoporosis).	
892	No. 84	80	84	10	3	29	Negative.	Negative.	Negative.	3.2
908		50	50				"	"	"	
901		40	30				"			
909		50	44				"	Negative.	" (slight osteoporosis).	
1075	No. 84	70	80	3	3	23	Negative.	Negative.	Negative.	
1076		60	64				"	"	"	
1077		50	68				"	"	"	
1078		30	38				" (?).	"	Almost negative.	
1071	No. 84	40	54	4	3	23	Negative.	Negative.	Negative.	
1072		40	50				"	"	" (slight osteoporosis).	
1073		42	50				"	"	"	
1074		36	48				"	"		

902	No. 84	50	50	10	3	23	Negative.	Negative.	Negative.	4.0
903		44	60				"	"	"	
904		50	50				"	"	"	3.65
905		44	50				"	"	" (osteoporosis).	
1083	No. 84	50	50	3	3	18	Negative.	Negative.	Almost negative.	
1084		50	50				"	"	Negative.	
1085		48	54				Very slight.	Very slight.	Minimal (healing).	
1086		50	54				Negative.	" "	Almost negative.	
1079	No. 84	50	54	4	3	18	Negative (?).	Negative.	Negative.	
1080		50	40				"	"	" (marked osteoporosis).	
1081		50	60				" (?).	"	Almost negative.	
1082		50	50				"	"		
933	No. 84	50	50	10	3	18	Negative.	Negative.	Negative.	
934		50	50				"	"	" (osteoporosis).	
935		48	50				"	"	"	
936		50	50				"	"	"	

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EXPLANATION OF PLATE 43.

FIG. 1. Rat 859 (white). 31 days on Diet 84. Daily exposure to the mercury vapor quartz lamp for 1½ minutes at a 3 foot distance. Rib: The zone of proliferating cartilage is normal. The zone of preparatory calcification (Pr_1) averages four cells in depth; matrix calcified. Complete calcification of spongiosa (*Sp*) and cortex (*Co*). No visible osteoid. No rickets. Decalcified in Müller's fluid for 5 days. Hematoxylin-eosin.

FIG. 2. Rat 853 (black). 31 days on Diet 84. Daily exposure to the mercury vapor quartz lamp for 1½ minutes at a 3 foot distance. Rib: The zone of preparatory calcification (Pr_1) is almost wholly free from calcium, and is greatly increased in depth and prolonged into the metaphysis. There is great excess of perichondral (*Pc*) and subchondral (*Sc*) osteoid. Marked rickets. Decalcified in Müller's fluid for 5 days. Hematoxylin-eosin.

FIG. 3, *a* and *b*. (*a*) Radiogram of the knee joint from a black rat (No. 853), showing marked rickets with fracture of the tibia. (*b*) Radiogram from a white rat (No. 859), showing a normal epiphyseal line.

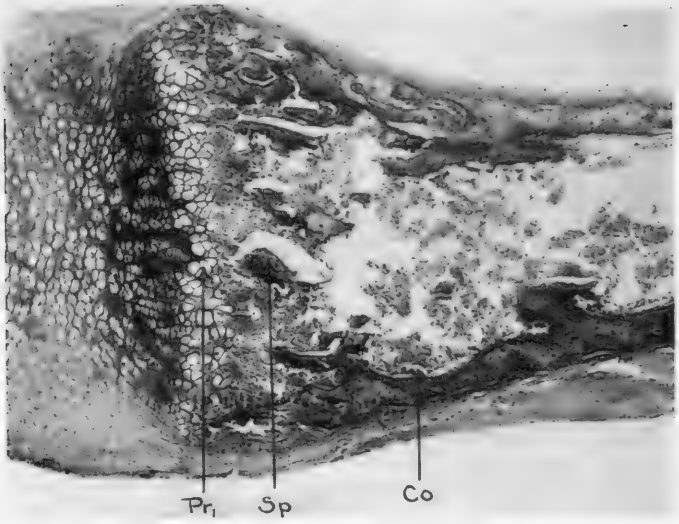

FIG. 1.

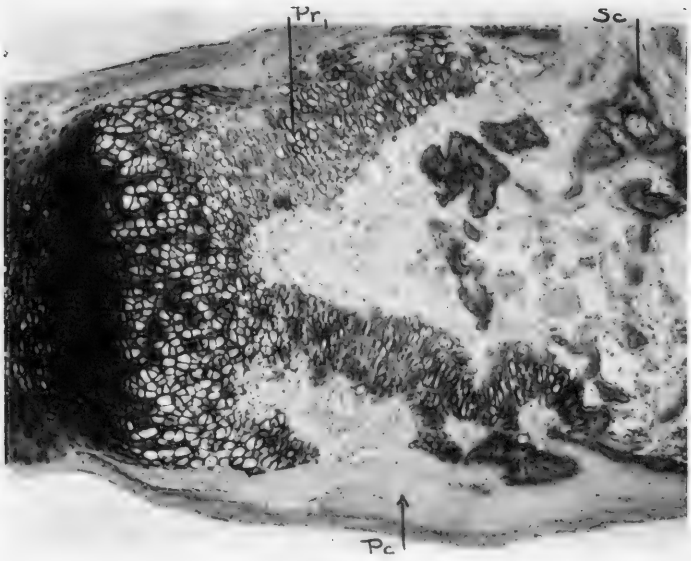

FIG. 2.

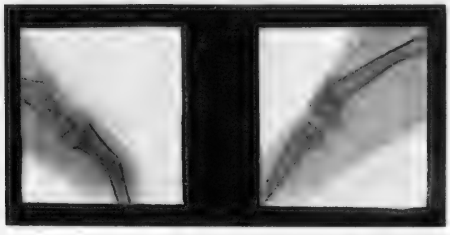

FIG. 3, *a*. FIG. 3, *b*.

(Hess, Unger, and Pappenheimer: Experimental rickets. VII.)

EXPERIMENTAL RICKETS IN RATS.

VIII. The Effect of Roentgen Rays.

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Plates 44 and 45.

(Received for publication, May 29, 1922.)

In view of the fact that the rays of the sun, of the mercury vapor lamp, and of the carbon arc lamp are able to protect rats from rickets, it seemed worth while to test the protective value of soft Roentgen rays. These experiments have been briefly referred to in another connection (1). As far as we are aware, the only report bearing on this subject is that of Huldschinsky (2), who treated a 3 year old rachitic child with rays from a tube having a hardness of 2.5–3° W., and observed calcification after eighteen irradiations in the course of 2 months. This author refers to a previous attempt at Roentgen ray therapy carried out by Winkler in 1920.

In addition to these preventive tests, a series of rats was subjected to massive doses of Roentgen rays, with the object possibly of damaging the cells to such an extent as to lead to the development of rickets. Many of the French writers, and especially Marfan (3) have held the opinion that changes in the bone marrow are of primary importance in the pathogenesis of rickets.

Table I shows the results of the protective experiments. The animals were on a rickets-producing diet, except that in one experiment 25 mg. of K_2HPO_4 per 100 gm. of diet were added in order to compensate for a supposed deficiency of phosphorus in the flour, and bring the diet up to the standard of 86 mg. per 100 gm. It will be noted that all the animals developed rickets, in spite of the irradiation, a result quite the contrary to that obtained when the mercury vapor or the carbon arc lamp was employed. This failure came about when the duration of the exposure was 15 minutes, and the distance of the

TABLE I.

Roentgen Ray Radiation. Attempted Protection from Rickets.

Rat No.	Diet.	Weight.		X-ray radiation.		Rickets.			Inorganic P per 100 cc. of blood.
		Initial.	Final.	Duration.	Type.	Radiogram.	Pathological examination.		
							Gross.	Microscopic.	
		gm.	*gm.*	*min.*					*mg.*
684	No. 84*	44	52	None (control).		Moderate.	Rickets.	Moderate.	3.2
685		50	62			"	"	"	
678	No. 84	44	50	15	5 milliamperes, ¾ inch spark-gap, 8 inch distance, no filter.	Moderate.	Rickets.	Moderate.	2.4
680		40	40			"	"	"	
679		40	40			"	"	"	
681		46	55			"	"	"	1.8
683		34	40			"	"	"	
682		44	46			"	"	"	
795	No. 84 + 25 mg. of K_2HPO_4.	52	54	2	15 milliamperes, ¾ inch spark-gap, 36 inch distance, † no filter.	Marked.	Rickets.	Moderate.	3.2
796		52	54			"	"	"	3.33
797		48	50			"	"	"	3.5
798		56	62			"	"	"	

* Patent flour..... 95.0 per cent.
Calcium lactate..... 2.9 per cent.
Sodium chloride..... 2.0 per cent.
Ferric citrate..... 0.1 per cent.

† 18 inches the 1st week.

Coolidge tube 8 inches, as well as when the period was shortened to 2 minutes and the tube was placed at a distance of 3 feet. A ¾ inch spark-gap, 15 milliamperes, and a universal Coolidge tube without filtration were used. The radiographs of the epiphyses at the knee joint showed the changes typical of the rickets which comes about on this diet. This was true likewise of the histological sections. The cartilage was wide and irregular, excess of osteoid was present, and there was no evidence that the picture had been altered by the Roentgen ray exposures (Figs. 1 and 2).

A series of animals was next subjected to very large doses of Roentgen rays. For this purpose we employed a universal Coolidge tube, at 8 inches distance, with 3 mm. of aluminum as a filter, a spark-gap of 6 inches, and 5 milliamperes of current. The exposures were for periods of 3 and 6 minutes. The dietary was No. 85, which consists of No. 84 with 75 mg. of phosphorus per 100 gm. of diet added. This diet is just sufficient to prevent the occurrence of rickets, but contains little phosphate in excess, so that if the intensive irradiation tended to produce rickets, it should be rendered evident on this diet. In no instance, however, did rickets develop (Table II)—merely the osteoporosis which, in our experience, is regularly produced by Diet 85. Fig. 3 illustrates the histological structure of the rib of Rat 837, and as far as the bony and cartilaginous structures are concerned, presents the typical picture encountered on this diet. The protocol of this animal is given in Table III.

Of particular interest are the changes observed in the blood at the time of necropsy and the histological alteration of the bone marrow. There was marked anemia, a reduction of the red cells by fully one-half; the control rats on the same diet had 5,120,000 to 6,500,000 red cells per c.mm. whereas the rats treated with Roentgen rays had 1,500,000 to 2,500,000 red cells. The hemoglobin averaged about 37 per cent in contrast to 79 per cent in the control animals. One of the striking features was the leucopenia which came about; the total number of leucocytes of the four rats was, respectively, 2,600, 2,100, 1,000, and 1,600 compared with an average of about 12,000 in the control rats. The diminution was most marked in the lymphocytes. This lymphopenia, following large doses of Roentgen rays, has been emphasized by Murphy and Morton (4) and their associates, who

TABLE II.

Roentgen Ray Radiation. Attempted Production of Rickets.

Rat No.	Diet.	Weight.		X-ray radiation.		Rickets.			Inorganic P per 100 cc. of blood.
		Initial.	Final.	Duration.	Type.	Radiogram.	Pathological examination.		
							Gross.	Microscopic.	
		gm.	*gm.*	*min.*					*mg.*
838	No. 85*	46	38	6	5 milliamperes, 6 inch spark-gap, 8 inch distance, 3 mm. aluminum filter.	Negative.	Negative.	Negative (osteoporosis).	
839		40	50			"	"	"	
840		50	50			"	"	" (osteoporosis).	
841		44	40			"	"	" "	
834	No. 85	46	58	3	5 milliamperes, 6 inch spark-gap, 8 inch distance, 3 mm. aluminum filter.	Negative.	Negative.	Negative.	4.36
835		40	50			"	"	"	
836		50	58			"	"	"	4.60
837		50	40			"	"	"	
831	No. 85	36	40	None (control).		Negative.	Negative.	Negative.	4.26
832		42	48			"	"	"	
833		36	60			"	"	"	

* Diet 85 is No. 84 plus 75 mg. of P added as K_2HPO_4.

found it to be the first change following radiation by Roentgen rays or by radium. The inorganic phosphate of the blood, 4.26 to 4.36 mg. per 100 cc., is approximately the percentage usually obtained in rats on this diet.

The bones showed no rachitic changes by radiograph during life, or by gross or microscopic examination after death. Fig. 3 illustrates the costochondral junction of Rat 837. It will be seen that it shows

TABLE III.

Protocol of Rat 837.

Date.	Weight.	Diet.	Remarks.
1921	*gm.*		
Dec. 17	50	No. 85	3 min. irradiation at 8 inches with filters.
" 19	50		
" 23	40		No irradiation Dec. 20 to 22, 24, and 26.
" 26	40		
" 30	44		
1922			
Jan. 3	54		No irradiation Jan. 1 and 2.
" 9	44		Jan. 10. Radiograph of epiphyses negative.
" 13	40		
" 16	40		
" 19			Killed for necropsy.
			Inorganic P of blood 4.60 mg. per 100 cc. (pooled with Rat 836). Hemoglobin 25 per cent; erythrocytes 1,250,000; polynuclear leucocytes 40 per cent; small mononuclears 52 per cent; large mononuclears 8 per cent.

none of the changes associated with rickets; the rows of cartilage cells are not increased in number, the matrix is well calcified, and there is no increase in osteoid tissue. The high power photograph (Fig. 4) is of especial interest. Here we note a marked alteration of the marrow. The specific marrow elements are replaced largely by edematous fatty tissue, containing cells resembling pale fibroblasts. Mitotic figures are occasionally seen in these cells. In other words, there is evidence of extreme exhaustion of the marrow.

CONCLUSION.

Rats on a low phosphorus diet cannot be protected from the development of rickets by exposures to soft Roentgen rays such as were employed in this study.

Furthermore, rats on a diet containing phosphate in an amount adequate to prevent rickets were not rendered rachitic by exposure to massive doses of Roentgen rays of an intensity sufficient to produce marked destruction of the blood-forming cells of the marrow.

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2. Huldschinsky, K., *Z. orthop. Chir.*, 1922, xlii, 240.
3. Marfan, A., Maladies des os, Paris, 1912.
4. Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800.

EXPLANATION OF PLATES.

PLATE 44.

FIG. 1. Rat 795. Diet 84 plus 25 mg. of K_2HPO_4 per 100 gm. of diet. 21 days on diet. Daily exposure to Roentgen rays (see Table I). Rib: Marked rickets. There is no evidence of prevention or healing under the influence of the Roentgen ray treatment. The greatly widened zone of preparatory calcification (Pr_1) is free from calcium in all portions. There is no recent calcium deposition in the perichondral osteoid (*Pc*) or in the osteoid of spongiosa (Sp_1) or cortex (*Co*). Decalcified in Müller's fluid for 5 days. Silver-nitrate-safranine.

FIG. 2. Rat 680. 21 days on Diet 84. Daily treatment with Roentgen rays (see Table I). No curative or preventive effect. The rib shows the usual typical rickets observed on this diet. Decalcified in Müller's fluid for 5 days. Silver-nitrate-safranine.

PLATE 45.

FIG. 3. Rat 837. 33 days on Diet 85. Intensive exposure to Roentgen rays (see Table II). Section of rib, low power. The zone of preparatory calcification (Pr_1) does not exceed five cells in depth. The matrix is densely calcified. The trabeculæ of the primary spongiosa (Sp_1) are short and irregular, and not surrounded by well formed osteoblasts. The cortex (*Co*) is relatively thick, and a distinct osteoid border is not visible, either about the cortex or the spongiosa. The marrow (*M*) is almost depleted of blood-forming elements, being composed of a loose edematous fibrous and adipose tissue, with congested blood vessels. Decalcified in Müller's fluid for 5 days. Hematoxylin-eosin.

FIG. 4. Rat 837. Bone marrow, showing depletion of blood-forming cells. × 420.

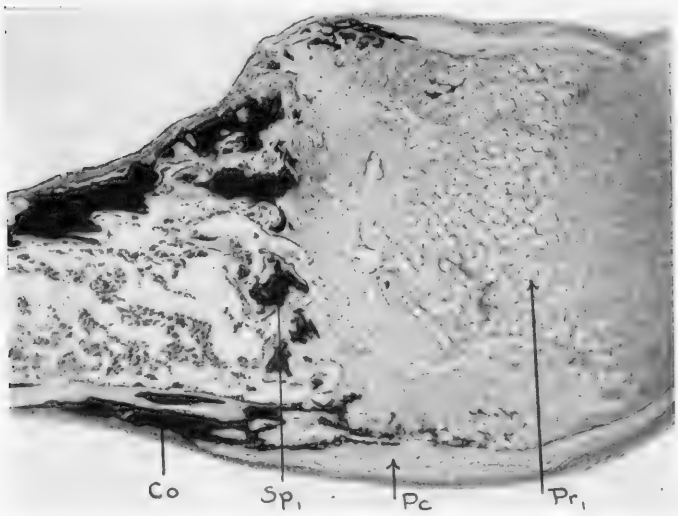

FIG. 1.

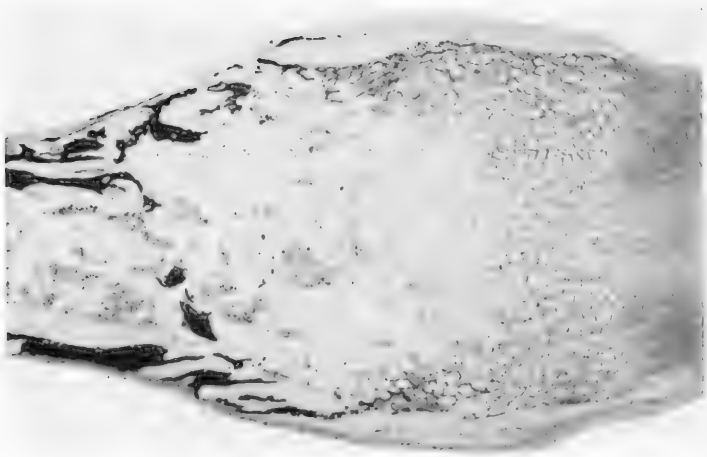

FIG. 2.

(Hess, Unger, and Steiner: Experimental rickets. VIII.)

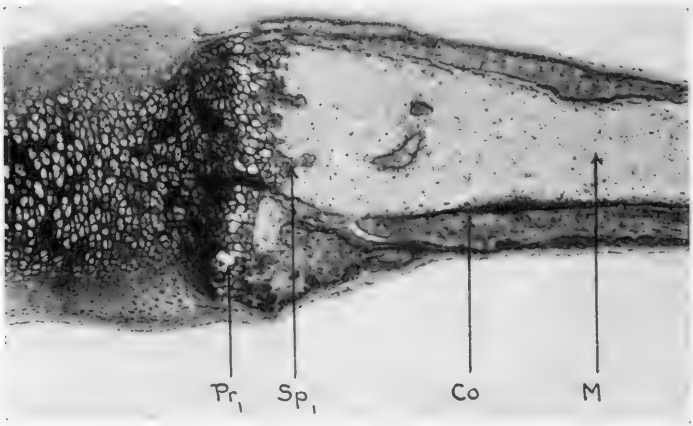

FIG. 3.

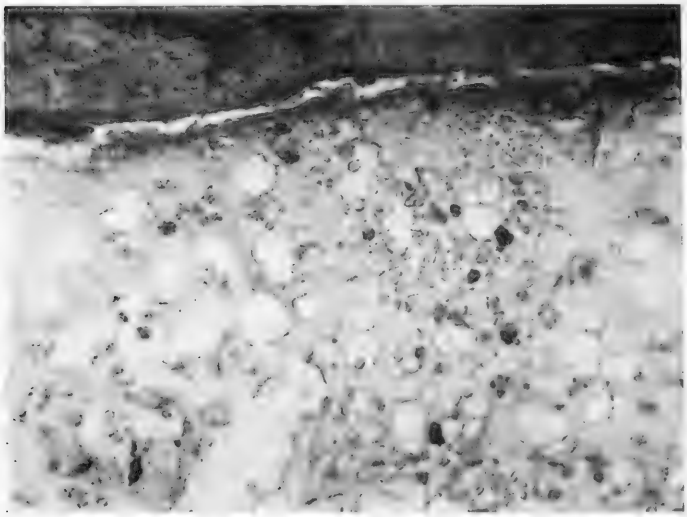

FIG. 4.

(Hess, Unger, and Steiner: Experimental rickets. VIII.)

COW SERUM AS A SUBSTITUTE FOR COLOSTRUM IN NEW-BORN CALVES.

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(Received for publication, May 31, 1922.)

In a former communication[1] it was shown that if new-born calves are divided in two groups, one of which receives colostrum from the dam and the other not, and if both groups are removed promptly from the surroundings of the herd, placed in individual stalls, and fed raw milk, the calves fed colostrum survive and develop as normal calves, while of the calves from which colostrum has been withheld the greater number die within the 1st week as a result of the invasion and multiplication of intestinal bacteria, chiefly *Bacillus coli*, throughout the body. Those that survive may develop lesions in or about certain joints, more rarely foci of sclerosis in both kidneys.

EXPERIMENTAL.

The rapid absorption into the blood of agglutinins towards *Bacillus abortus* ingested in the colostrum[2] indicated that the immunity of the calves receiving colostrum is due to the protective antibodies which tend to accumulate in the colostrum up to the time of parturition. If this inference is true the blood of the adult cow should also contain these various antibodies and might protect calves not fortified by colostrum. To test this hypothesis, three groups of calves were treated with the blood serum of the same cow in slightly different ways.

Calves of which it was certain that they had not suckled the dam were brought to the animal quarter of the Department soon after birth, thoroughly washed with water of about body temperature, and

[1] Smith, T., and Little, R. B., *J. Exp. Med.*, 1922, xxxvi, 181.

[2] Little, R. B., and Orcutt, M. L., *J. Exp. Med.*, 1922, xxxv, 161.

dried by rubbing with towels. Each calf was placed in a separate stall and fed milk from cows in about the middle of the lactation period. The milk was at first fed from bottles or other receptacles through nipples and after the 1st week from pails.

The serum used throughout was obtained from the same cow at intervals during the experiment. The animal was a grade Guernsey, in milk when purchased from the Middle West November, 1920. She was chosen at random mainly because easily controlled. The blood was drawn from a jugular vein under aseptic conditions and allowed to clot. The serum was withdrawn after 2 days, passed through a Berkefeld filter, and stored at 38–40°F. without an antiseptic.

No efforts beyond isolation in individual stalls and ordinary cleanliness were made in rearing the calves. Owing to the nature of the experiment and the many uncontrolled factors entering into it, it has seemed best to record certain details which may be of significance in the interpretation of results. These are given in the notes on the individual calves.

The first group of five calves received serum both into the jugular vein and the subcutis immediately after the cleansing bath. On the following day serum was again injected into the subcutis. The following notes furnish the chief details.

Calf 901.—Guernsey bull, born Mar. 10, 1.15 p.m., of a native cow in her second pregnancy. Removed from dam immediately after birth. Received at Department 2.45 p.m. Washed in water at 100°F. containing a little soda. Dried off by gentle friction with bath towels. Calf quite weak after the cleansing. Kept warm with blankets. Receives into jugular vein 20 cc. serum and 20 cc. into subcutis in three different places. 5 p.m. Fed 1¼ lbs. milk. 11 p.m. Second feeding of ¾ lb. milk.

Mar. 11. Meconium discharged during the night. Calf received about 2 lbs. milk. Urine collected has a specific gravity of 1,008. Contains no abnormal products. At noon calf received into subcutis 20 cc. serum. Diarrhea started late today. Temperature normal. Total amount of milk taken about 4 lbs.

Mar. 12. Stools less fluid, light yellow. Temperature normal. Total milk taken about 6 lbs.

Mar. 13. Feces consistent, orange to yellow. 7½ lbs. of milk taken.

Mar. 16. Calf is fed from pail. Takes about 7½ lbs. of milk.

Apr. 12. Calf has progressed normally, taking slightly more milk each week. Today 12 lbs. consumed. Since Apr. 4 and 5 a little hay and grain were placed in the stall each day.

Apr. 13. Calf killed today. Weight 102 lbs. Autopsy negative with exception of cecum in which there is a wide meshed pigmentation over the submucous vessels.

Calf 538.—Holstein bull, born 12.40 p.m., Mar. 16, of a cow in her third pregnancy. Brought to Department at 1.15 p.m. Washed in the same way as described for Calf 901. 2 p.m. Fed ¾ lb. of milk. 2.15 p.m. 18 cc. of serum injected into jugular vein and 22 cc. into subcutis. Fed milk at 5 and 10.30 p.m. Calf weak and able to stand only a short time.

Mar. 17. Begins to discharge meconium during the day. Takes milk from bottle readily, about 4 lbs. in all taken today. Stronger, moves about stall. Receives 20 cc. serum into subcutis at 3.30 p.m. Late in the evening calf was quite weak, scarcely able to stand. Extremities cold. Mucous membranes pale. Able to take only a little food at 11 p.m.

Mar. 18. Dies at 2.30 a.m. Weight after death 66 lbs. The autopsy showed no gross lesions to account for death. The fourth stomach contained over a liter of milky fluid and curds. The latter made up about one-third of the total. Mucosa was sprinkled over with deep and superficial petechiæ.[3] In the small intestine occasional congested areas. In the large intestine the longitudinal folds form dark red streaks of congestion and hemorrhage. Cultures containing bits of tissue from spleen, liver, and kidneys remain sterile. From several segments of the small intestine *B. coli* isolated as the chief type present.

Calf 485.—Guernsey heifer, born Mar. 24, 9.15 a.m., of a native cow in her third pregnancy. Removed immediately and brought to Department. Washed and dried. 10.27 a.m. Injected 20 cc. serum into jugular vein and 20 cc. into subcutis. Calf rather weak. 1.45 p.m. Receives the first dose of milk from bottle. Fed again at 5 and 11 p.m. Calf active. Rather dry meconium voided.

Mar. 25. Feces growing soft, liquid later in the day. In the evening the calf loses its activity and takes its milk more slowly. Feces very soft and somewhat fetid; light yellow in color. Took in all about 3 lbs. of milk.

Mar. 26. Has been scouring during the night and continues during the day. Fed five times today, the calf taking only small amounts. Very weak late at night.

Mar. 27. Calf very weak, unable to get up. Takes a few ounces of milk. Temperature 37.2°C. Extremities growing cold. Chloroformed later in the afternoon and refrigerated. Calf weight 49 lbs.

Mar. 28. Autopsy shows the following abnormalities. Fourth stomach nearly empty. Minute hemorrhages present in small number. Upper small intestine congested in streaks and patches. Fat stasis indicated by whitish club-shaped villi. Epithelium contains fat granules. The rest of small intestine deeply congested in streaks, patches, and continuously for some distance. Longitudinal streaks of congestion in large intestine. Kidneys uniformly congested. Lungs mottled with pale red patches of atelectasis.

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[3] Smith, T., and Little, R. B., *J. Exp. Med.*, 1922, xxxvi, 184.

Agar slant containing a bit of kidney tissue remains sterile. One plus spleen tissue develops only around the bit of tissue. Subcultures from this contain besides *B. coli* a small diplococcus (enterococcus). A tube containing a bit of liver tissue also develops growth only about the tissue. Subcultures show only *B. coli*. The same type of *B. coli* was isolated from two segments of the small intestine in pure culture. This type is characterized by marked viscidity of the growth and large involution forms. Occasional motile elements detected.

Calf 767.—Holstein heifer, first calf of native cow, born 11.15 a.m., Mar. 24. Removed at once from dam, taken to Department, washed, dried, and blanketed. 11.55 a.m. Receives 20 cc. serum into a jugular vein and 20 cc. into the subcutis. Fed first milk at 3.15 p.m., next at 11.10 p.m. Calf, weak after cleansing, was stronger at night.

Mar. 25. Voids a large amount of dark greenish meconium. At 11 a.m. 20 cc. serum injected into subcutis. Fed three times today, in all about 3 lbs. of milk.

Mar. 26. Feces becoming softer and fluid late in the day, yellowish in color. Fed three times, in all 3½ lbs. of milk.

Mar. 27. Weaker today. Feces still fluid. Took only 3½ lbs. of milk.

Mar. 28. Calf quite weak. Feces still soft and yellowish. Takes 3½ lbs. of milk in all.

Mar. 29. Calf unable to stand. Has to be held when taking food. Fed four times. Took 3½ lbs. in all.

Mar. 30. Calf begins to improve. Feces less fluid. Takes 4 lbs. of milk. Gets up and moves about stall.

Apr. 19. Since Mar. 30 the calf has improved steadily and continued to take larger quantities of milk. Today 11 lbs. taken. The calf is emaciated and shows stiffness, without localized joint lesions, in the hind limbs. For several days (Mar. 30 and 31) there was a slight conjunctivitis.

Apr. 20. Calf killed today. Weight 78 lbs. No lesions were found. Cultures made with bits of spleen, liver, kidneys, and several drops of fluid from both knee and tarsal joints remained sterile.

Calf 948.—Female Holstein calf, born of a heifer in her first pregnancy, at 1.30 p.m., May 3. Calf weighs 88½ lbs. Labor difficult and calf delivered in the last stage by traction. Removed at 3.50 p.m. to the Department, washed, and dried. 4.40 p.m. 40 cc. serum injected into jugular veins and 20 cc. into subcutis. 7.50 p.m. First meal of 2 lbs. of milk. Fed again at 10.30 p.m. and 1 a.m.

May 4. Receives into subcutis 40 cc. serum. Fed four times today, the last meal at 10 p.m. Drank in all about 8 lbs. of milk.

May 5. Feces becoming soft and observed to be very watery and ejected in a stream at 1.15 p.m. Calf weak, growing thin. Eyes sunken. Has taken only about 6 lbs. of milk today.

May 6. Calf weaker; still able to stand but gait unsteady. Feces fluid during the day, light yellow in color. Takes in all about 4 lbs. of milk.

May 7. Temperature is 40.2°C. this morning. Feces continue fluid. Calf unable to stand. Takes but little milk when fed at 11 a.m. 48 cc. of cow serum injected subcutaneously. Calf dies this afternoon. Body chilled with cold water and refrigerated till next morning.

May 8. From the autopsy notes the following may be mentioned. The fourth stomach contains a small amount of a thick, viscid material plus small curds. Hemorrhagic blotches scattered through leafy portion. In the small intestine there is moderate congestion in patches above which increases in intensity downwards. In the ileum the mucosa is dark red and overlaid with a surface film of orange-colored mucoid material suggesting superficial necrosis. The large intestine is congested along summits of the longitudinal folds. Films from small intestine contain many rods, like *B. coli*.

Bits of spleen and kidney in agar slants remain sterile. In the liver tube one colony of *B. coli* appears. The dilution cultures from the small intestine contain colonies of *B. coli* only.

It will be noted that two of the five calves died in about 48 hours after birth. A third was chloroformed, moribund, when $3\frac{1}{4}$ days old. A fourth calf was very sick when 3 days old, but recovered. The fifth calf passed through a period of diarrhea lasting nearly 2 days, but continued to take its food and made a good recovery.

Of the three calves that died, the organs of one were sterile. In the second case, the kidneys were sterile but growth appeared about the bit of liver and spleen tissue, indicating the presence of very few bacteria as compared with the condition of the same organs in controls not receiving either colostrum or serum. In the third both spleen and kidneys were sterile and the liver tube contained one colony.

A second group of five calves received the bovine serum not only intravenously and subcutaneously but also in the milk of the first two meals. The following notes of these cases supply the individual data.

Calf 768.—Holstein bull, born 5.45 p.m., Apr. 10, of a native cow in her third pregnancy. Weighs between 85 and 90 lbs. Removed immediately to the Department, washed, and dried. 7.20 p.m. 20 cc. serum injected into subcutis and 20 cc. into jugular vein. 10.10 p.m. Fed nearly 2 lbs. of milk plus 60 cc. serum mixed with it. 2.30 a.m. Fed milk and 60 cc. serum.

Apr . 11. Considerable meconium passed during the night. 20 cc. serum injected subcutaneously at 9.45 a.m. Takes in all 8 lbs. of milk today.

Apr. 12. Diarrhea during night and this morning. Takes food as usual.

Apr. 13. Feces soft and grayish yellow. Calf takes 8 lbs. of milk today.

Apr. 30. The calf has been progressing normally since the preceding date. Took 11 lbs. of milk today.

May 1. Killed and found normal.

Calf 905.—Holstein bull, born Apr. 7, at 10.30 a.m., of a native cow in her fourth pregnancy. Weighs 114 lbs. Taken to the Department immediately after birth, washed, and dried. 11.40 a.m. 20 cc. serum injected into a jugular vein and 20 cc. into subcutis. 2 p.m. Received first meal of milk, 1½ lbs. plus 60 cc. serum. 5.40 p.m. Fed the same quantity of milk and serum. 10.35 p.m. Fed 2 lbs. of milk.

Apr. 8. Receives 20 cc. serum into subcutis at 10 a.m. Considerable dark greenish meconium discharged today. Feces soft late at night.

Apr. 9. Feces still fluid. Considerable blood discharged at one time during defecation. Calf otherwise active and hungry. Takes in all today 9 lbs. of milk.

Apr. 11. Feces still slightly tinged with blood.

Apr. 13. Takes in all 11 lbs. of milk. Lameness in right fore leg without local signs.

Apr. 14. Lame in right hind leg. Hock and ankle swollen. Apr. 15. Temperature slightly elevated (39.6°C.). Blood still voided on feces. Apr. 17. Stiffness and lameness have disappeared. Calf very active. Apr. 23. Takes 12 lbs. milk. Apr. 28. Slight febrile disturbance. Apr. 29. Normal today.

May 1. Killed for food. Organs normal.

Calf 906.—Holstein female, born 9.30 a.m., Apr. 11, first calf of a native heifer. Calf small, weighing 65 lbs. Received at Department at 10.30 a.m., washed, and dried. At 11.10 a.m. 20 cc. serum injected into a jugular vein and 20 cc. into the subcutis. 2 p.m. Fed 2 lbs. of milk to which were added 60 cc. serum. 5.35 p.m. The same dose repeated. 10.30 p.m. Fed 2 lbs. of milk.

Apr. 12. Receives into subcutis 20 cc. serum. Meconium passed during the night. Calf active and takes its food well.

Apr. 13. Feces liquid, greenish yellow. Calf otherwise normal. Apr. 15. Took 6½ lbs. of milk today. Apr. 18. Took 8 lbs. of milk today. Apr. 21. Took 11 lbs. of milk today.

May 1. Killed for food. Organs normal.

Calf 932.—Holstein bull, born of a western cow, at 7.20 a.m., Apr. 17. Calf weighs 100 lbs. Taken to Department, and washed and dried. 10 a.m. Receives 20 cc. serum into a jugular vein and 20 cc. into the subcutis. Refuses food at noon. Takes 2 lbs. of milk at 4.50 p.m. to which 60 cc. serum had been added. At 10.15 p.m. this dose was repeated.

Apr. 18. Receives 20 cc. serum into subcutis. Calf constipated.

Apr. 19. Feces fluid, light yellow in color, later in day nearly colorless. Takes milk normally.

Apr. 20. Feces still soft.

Apr. 21. Takes 9 lbs. of milk in all. Some blood discharged on feces.

Apr. 24. Takes 11 lbs. of milk. Appears entirely normal. Apr. 26. Took 11 lbs. of milk today. Some blood passed on feces. Apr. 27. Blood still passed with feces. Apr. 29. Feces normal.

May 1. Killed for food. Normal.

Calf 953.—Guernsey female, born May 20, 9.15 a.m., of a cow in her fourth pregnancy. Calf rather thin, weighs 70 lbs. Received at the Department at 10.15 a.m. Simply dried off, without preliminary washing, with bath towels. Calf able to stand and move about. 10.40 a.m. Received into jugular vein about 39½ cc. of serum and 20 cc. into subcutis. 11 a.m. Fed 2 lbs. of milk plus 55 cc. serum. 4.20 p.m. The same feeding repeated. 11.15 p.m. Fed 2 lbs. of milk.

May 21. Calf very eager for food. 2 lbs. of milk taken. 9.30 a.m. 20 cc. serum injected into subcutis. Urine collected at this time. No fluid feces observed today. Fed in all 6 lbs. of milk.

May 22. Took 6½ lbs. of milk today. Feces passed resemble meconium. No diarrhea.

May 26. Slight diarrhea today. For several days past, the feces have been rather light in color but of normal consistency. Today the calf took 8 lbs. of milk.

May 27. Feces slightly softer than normal, light yellow. Urine collected early shows 0.06 per cent albumin (Esbach), specific gravity 1,010, slightly acid, pale yellowish, and faintly clouded. Sediment slight, containing some short hyaline casts filled with amorphous granules.

May 28. Feces normal. Only a trace of albumin in urine today.

May 30. Normal.

Two of these five calves manifested a slight diarrhea associated with hemorrhage which soon disappeared. Four were killed when 14, 21, 24, and 20 days old respectively. No abnormalities were noted. The fifth is still alive.

A third group of five calves received the serum in the milk of the first and second meals. Whatever protection was afforded by it had to be exerted through the digestive tract.

Calf 772.—Small Guernsey calf, born at 12.10 p.m., Apr. 19, of a native heifer in her first pregnancy. Weight 50 lbs. Taken away before it had suckled the dam, received and cleansed at 2 p.m. Calf very weak, unable to stand. Large amount of dark, soft meconium voided. 4.45 p.m. First feeding of 1 lb. of milk to which 100 cc. of serum had been added. 10.30 p.m. Calf fed 1½ lbs. of milk to which 100 cc. of serum had been added. Still unable to get up.

Apr. 20. Calf continues weak. Fed milk at four intervals during the day. During the last feeding at 10.15 p.m. the calf failed to take its food. Meconium voided during the day, now yellowish.

Apr. 21. Calf died at 4 a.m. Considerable diarrhea preceding death. Autopsy next morning presents the following abnormalities.

Fourth stomach contains a handful of white, rather firm curds and some milky fluid. Mucosa normal. Mucosa of small intestine deeply congested throughout. Large intestine less so.

Endocardium of left ventricle raised in the form of linear ridges by subendocardial hemorrhages.

Kidneys deeply and uniformly congested throughout. Urine taken from bladder shows only a trace of clouding when slightly acidified with acetic acid and heated to boiling.

Cultures prepared by adding bits of tissue from spleen, liver, and kidneys to slanted agar remained sterile. Cultures from successive dilutions of contents of small intestine contain colonies of *B. coli* and some colonies of smaller size, probably enterococci.

Calf 935.—Holstein female, weighing about 85 lbs., born 6.30 a.m., Apr. 21, of a heifer. Calf not allowed to suckle dam. Received at Department at 9.40 a.m., washed, dried, and blanketed. 11.40 a.m. Received first meal of 2 lbs. of milk plus 100 cc. serum. 4.30 p.m. The same dose repeated. 10.20 p.m. Fed 2 lbs. of milk.

Apr. 22. Dark meconium passed during night. Feces grew liquid towards noon, yellowish in color. Calf took 4½ lbs. of milk in all today.

Apr. 23. Scouring a little early in the day. No movements later. Fed four times and took about 7 lbs. of milk.

Apr. 24. Calf improving and quite active. Fed three times, in all about 7 lbs. of milk. Urine caught is normal, specific gravity 1,012.

May 15. Has been taking 15 lbs. of milk daily. In good condition. Sold to butcher today. The organs appear normal with exception of kidneys. On close scrutiny there are seen in many of the lobes groups of feebly accentuated grayish areas less than 1 mm. in diameter. On section these correspond to narrowly triangular pale areas passing radially to medulla.

Calf 936.—Large Holstein bull, weighing about 100 lbs., born 9.20 a.m., Apr. 21, of a heifer (first pregnancy). Owing to its large size calf had to be delivered by traction in the last stage. Calf kept from suckling dam and taken to Department at 11 a.m., washed, dried, and blanketed. Milk refused by calf until 4.35 p.m. when it took 1¾ lbs. plus 100 cc. serum. A little of this mixture was lost in feeding. 10 p.m. Fed about 2 lbs. of milk plus 100 cc. serum. The calf has been rather weak all day.

Apr. 22. Large amount of meconium passed during the night. Feces becoming fluid during the day. Takes but little milk.

Apr. 23. Calf more active and feces firmer, yellowish in color. Fed four times, taking in all about 8 lbs. of milk.

Apr. 24. Calf active. Feces still soft and light yellowish in color.

Apr. 29. Slight swelling and lameness of left tarsal joint. Left fore leg shows lameness but no recognizable swelling. Receiving 12 lbs. of milk.

May 13. Calf not quite normal. Drank milk slowly. Evening temperature 39.9°C.

May 14. Condition unchanged. Takes 12 lbs. of milk. Temperature 39°, 39.8°, and 40°C.

May 15. Temperature 40°C. Killed today. The only abnormality found is in the kidneys. Multiple foci varying from 2 to 8 mm. in diameter; from two to twelve in a lobe. These are firm, smooth, and glistening white on section. They are broadly triangular with base at the surface and apex at junction of cortex with medulla. No reactive zone around them.

Calf 940.—Large Holstein heifer, weighing about 110 lbs., born 2.20 p.m., Apr. 25, of a native cow in her second pregnancy. Treated in the usual way. Calf weak and unable to stand. 4.40 p.m. Received the first food, 2 lbs. milk plus 100 cc. serum. 11 p.m. Fed 2½ lbs. milk plus 100 cc. serum. Dry meconium discharged.

Apr. 26. Feces became fluid towards afternoon, light yellow in color. Fed four times today. Took in all 8 lbs. of milk.

Apr. 27. Diarrhea checked. Calf active and hungry.

Apr. 29. Takes 11 lbs. of milk.

May 3. Takes 14 lbs of milk.

May 15. This calf has been normal, taking 17 lbs. of milk since May 11. Killed today. Organs normal.

Calf 951.—Holstein female, born May 8, at 4.30 p.m., of a heifer. Parturition normal. Calf weighs 77 lbs., rather thin. Taken to the Department at 5 p.m., washed, dried, and removed to a single stall. 7.10 p.m. Fed the first meal of milk plus 100 cc. cow serum. 11.40 p.m. Received 2 lbs. of milk plus 120 cc. serum. Moves actively about stall.

May 9. Fed 2 lbs. of milk plus 120 cc. serum. Calf takes the mixture readily. This is the last serum fed. Milk taken at 5 and 10 p.m. Calf has been constipated. Meconium passed during the night.

May 10, 8.15 a.m. Temperature slightly elevated (39.4°C.). Diarrhea sets in. Calf feeds slowly and shows signs of depression. Grows weaker during the day while diarrhea continues. Feces tinged with blood. Temperature at 11 p.m. 39.6°C.

May 11. Calf dies at 5 a.m. Chilled with cold water and refrigerated until 9.30 a.m. Of the autopsy notes, the following are significant. Fourth stomach contains a small amount of milky fluid and some curds, totalling a mass 4 to 5 cm. in diameter. Mucosa covered with a thin layer of mucus. Minute hemorrhagic points, deep, faded, and superficial appear in two dense groups covering a total area about 10 cm. in diameter. Small intestine congested throughout in streaks and patches, the congestion increasing downwards. Large intestine congested along longitudinal folds.

Medulla of kidneys deeply congested. Bladder distended with 775 cc. urine. No obstruction found to account for the retention. Specific gravity of urine 1,010. Faintest trace of albumin present.

Marked fatty degeneration of liver cells. Orange pigment granules present.

Bits of liver and kidney in agar tubes remain sterile. In the spleen tube in place of *B. coli* about fifteen colonies of an enterococcus present. *B. coli* appears in cultures from the small intestine as in earlier cases.

It will be noted that two of this group died. One was small and weak from the start, unable to get up, and lived less than 2 days. The spleen, liver, and kidneys were sterile, however. Since the organs of all control calves thus far contained numerous bacteria, it may be assumed that the serum was responsible for this condition.

The second calf which died lived 2½ days. The liver and kidneys were sterile but in the spleen tube about fifteen colonies of enterococcus appeared.

Since in the former series[1] such a high percentage of calves from which colostrum had been withheld died, only two additional calves were treated in a similar way as controls during the period of this experiment. These died within 2 days. Spleen, liver, and kidneys were flooded with bacilli of the *Bacillus coli* type.

Calf 945.—Female, born of a native heifer Apr. 30, 10.20 a.m. Weighs 79½ lbs. Not allowed to suckle the dam and taken to the Department buildings at 10.40 a.m. Washed and dried and removed to a single stall. Calf fed at 1 p.m. Drinks with difficulty and takes only 10 ounces. 6 p.m. Takes about 1 lb. of milk very slowly. Fed again about 1½ lbs. at 10 p.m. During the day considerable meconium was discharged.

May 1. Takes about 8 lbs. of milk during the day. Bright and active early in the day. Feces become watery later in the morning and continue so during the day. Later tinged with blood.

May 2. Calf died during the night. Autopsied early this morning. Externally nothing abnormal. The fourth stomach somewhat dilated. It contains several firm white curds, the size of an egg, and considerable milky fluid. The entire mucosa has a deep red color. In the small intestine the mucosa is diffusely and deeply congested in the middle portion, in upper and lower segments less so. Films show many bacilli resembling *B. coli.*

Beneath endocardium of left ventricle there are many hemorrhages. The blood in general is in soft, dark clots, filling the large vessels with large molds. The liver contains much fat and orange pigment. The kidneys are uniformly congested to the tips of papillæ. Urine from bladder contains a trace of albumin; clear, yellow, specific gravity 1,032.

Films from spleen, liver, and kidney tissue contain scattering colon-like rods in every field. Cultures from these organs indicate presence of large numbers of bacteria. Only after one dilution of a bit of tissue in 6 cc. of bouillon are colonies

discrete on agar slants. The bacteria resemble *B. coli*. Dilution cultures from a segment of the middle zone of the small intestine show *B. coli* and enterococci.

Calf 947.—Holstein bull calf, delivered by mechanical help, May 3, 10 a.m. Presentation normal. Mother a native heifer. Weight of calf 86 lbs. Taken to Department at 11.50 a.m. and treated as the others. It was noted when cleansing calf that the umbilical cord had been torn away during delivery. Raw surface treated with boric acid. 2 p.m. Received first meal of 2 lbs. of mixed milk. Fed again at 5.20 and 10.20 p.m. Received in all 6 lbs. of milk.

May 4. Calf's feces becoming fluid. Calf growing dull. Respirations rapid and superficial. Refuses midday meal. Fed at 5 and 10 p.m. Calf growing weaker, takes but little food. Discharges continue watery.

May 5. Feeding continued at intervals during the night and day. Symptoms as yesterday. Calf grows weaker, takes but little food. Dies towards evening. Refrigerated until next morning.

The autopsy showed few differences between this case and Calf 945. There were many superficial hemorrhagic points in the fourth stomach. The small intestine and the kidneys showed very little congestion. The liver contained considerable fat and orange pigment. The bacteriology of the organs and the small intestine was almost identical with that of Calf 945 and a description of the cultures is therefore superfluous.

During this same period two calves were placed under observation which had been permitted to take colostrum. One of these died after $5\frac{1}{2}$ days. This was the first colostrum-fed calf to die in our hands. The other developed normally after a short period of diarrhea. The dead calf's organs were sterile.

Calf 949.—Holstein bull calf, weighing 99 lbs., born of a native heifer, May 3, 5.15 p.m. Labor prolonged from 11 a.m. Presentation normal. Traction applied to fore legs in the last stage. Calf allowed to suckle dam for 15 minutes, then removed to Department, washed, dried, and bedded in clean stall.

May 4. Fed 8 lbs. of milk in four doses. Calf constipated.

May 5, 10.30 a.m. Large amount of meconium and yellowish feces passed. Diarrhea started in afternoon. Respirations accelerated. Temperature 39.6°C. at 5 p.m.

May 6. Feces continue fluid. Light yellow in color. Calf appears sick. Temperature 40.1°C. at 10 p.m. Has been taking food regularly until night.

May 7. Diarrhea continues through the day. Calf growing thin. Temperature 40.2°C. at night. Took about 7 lbs. of milk today.

May 8. Calf growing weaker and unable to stand up and swallow its food late at night. Diarrhea continues.

May 9. Calf died at 4.30 a.m. Refrigerated until 9.30 a.m. Weight 76 lbs. From the autopsy notes, the following conditions are noteworthy.

The fourth stomach was distended with about 2 liters of a milky fluid and few curds. The mucosa was overlaid with mucus and congested. The small intestine was slightly congested above. The congestion increased downward and was severe in the ileum. The large intestine was congested along summits of longitudinal folds. The other viscera showed nothing unusual.

Tubes of agar containing bits of spleen, liver, and kidney remain free from growth. In dilution cultures of the middle region of the small intestine, *B. coli* was present as in preceding cases.

Calf 769.—Holstein bull calf, born Mar. 29, 8.15 a.m., of a native cow in her second pregnancy. Calf weighs about 100 lbs. 10.30 a.m. Placed with dam and allowed to suckle. Removed to Department at 1.30 p.m., washed, dried, and placed in a fresh stall. The cord had been torn off close to the abdominal wall. The exposed area dusted with boric acid powder. Calf appears strong and able to move about. Fed at 10.30 p.m. 1½ lbs. of milk.

Mar. 30. Feces become fluid during the day. Calf active. Takes in all 5½ lbs. of milk.

Mar. 31. Feces fluid and yellowish in color early, becoming less watery later in day. Milk as yesterday.

Apr. 1. Feces normal today. Calf takes 6 lbs. of milk.

Apr. 26. Calf has been normal in condition to date. Now taking 11 lbs. of milk.

Apr. 27. Killed today. No abnormalities found at autopsy.

During this same period of 2 months, twelve calves died of spontaneous scours in the herd from which our animals were taken. Two of these were autopsied.

Calf 904.—Born Mar. 31, of a native cow in her second pregnancy. Calf appeared well on Apr. 1. Diarrhea started Apr. 2. The calf grew weaker and died at noon Apr. 3. The autopsy brought out the following conditions.

Rumen contains a handful of shavings and some opalescent fluid. Similar fluid in fourth stomach totalling about 400 cc. No signs of milk or milk curds in the stomachs. The upper third of the small intestine is markedly congested, similarly the lowest third. Middle third hemorrhagic. The hemorrhages tend to raise the mucous surface into elevated streaks and patches. The large intestine is congested throughout in the form of close longitudinal lines. In the rectum the congestion involves the entire mucosa. Liver cells contain much fat. Subendocardial hemorrhages about papillary muscles of left ventricle.

Cultures show large numbers of *B. coli* in spleen, liver, and kidneys.

Calf 934.—Fourth calf of a native cow. Born Apr. 19. Died Apr. 20 at noon. The autopsy showed plenty of colostrum in the fourth stomach. Mucosa a uniform dark red. Small intestine is only moderately congested. There is marked fat stasis indicated by expanded, rather rigid villi full of fat. Urine taken from bladder clear, yellow, contains 0.3 per cent albumin (Esbach).

In this case spleen, liver, and kidneys are sterile. *B. coli* of the usual type isolated from contents of small intestine.

DISCUSSION.

The immediate object of the foregoing experiments was to determine whether colostrum was primarily protective against intestinal bacteria by replacing it by some easily absorbed substance containing the immune bodies found in colostrum. The substance chosen was cow serum.

If the results obtained for and against this assumption had been fairly close they could scarcely be regarded as of any value owing to the number of unknown and largely uncontrollable factors entering into such relatively crude tests. The results point, however, very decidedly in one direction—towards serum as a protective substitute for colostrum. In the first group of five calves receiving serum into a vein and the subcutis, three out of five died and a fourth was very sick. In the second group of five which received the same treatment plus serum fed in the milk none died. In the third group of five which received serum in the milk, two died.

In Table I are brought together certain data bearing on the milk fed and the serum administered. It will be seen that the milk came from three cows. In one case the 1st day's milk came from two other cows. No evidence can be adduced that the milk was responsible for the outcome. The same is true of the serum. The same cow was bled three times and the three lots of serum are designated A, B, and C. The particular lot and its age, counting from the day it was drawn from the cow, are recorded in the table. The age of the serum does not seem to figure in the results obtained. Among the group to which serum was fed, the two that died received the freshest serum of the entire lot.

The calves came from grade stock. Neither breed nor sex nor the number of the pregnancy can be drawn in as having any direct bearing on the outcome.

The results indicate that the cow serum introduced both by way of the blood and the digestive tract replaced colostrum successfully. Simply feeding the serum, though only partially successful, appears superior to injecting it. These figures become more favorable to the

TABLE I.

Calves Treated with Cow Serum in Place of Colostrum.

No.	Breed* and sex.	No. of pregnancy.	Date of birth.	Died. Age.	Killed. Age.	Cow's milk fed.	Age of serum used.	Mode of administration of serum.
				days	*days*		*days*	
901	G.; male.	2nd	Mar. 10		34	678 + 712	A—22	Injected.
538	H.; female.	3rd	" 16	1½		678 + 712	" 28	"
485	G.; "	3rd	" 24	3½		678 + 712	" 36	"
767	H.; "	1st	" 24		27	678 + 712	" 36	"
948	" "	1st	May 3	4		712 + 938	B—22	"
905	" male.	4th	Apr. 7		24	678 + 712	A—50	" and fed.
768	" "	2nd	" 10		21	600 + 897	" 53	" " "
						678 + 712		
906	" female.	1st	" 11		20	678 + 712	" 54	" " "
932	" male.	1st	" 17		14	678 + 712	" 60	" " "
953	G.; female.	4th	May 20		Normal May 30.	712 + 938	C—19	" " "
772	" male.	1st	Apr. 19	1¾		678 + 712	B— 7	Fed.
935	H.; female.	1st	" 21		23	678 + 712	" 9	"
						712 + 938		
936	" male.	1st	" 21		23	678 + 712	" 9	"
						712 + 938		
940	" female.	2nd	" 25		19	712 + 938	" 13	"
951	" "	1st	May 8	2½		712 + 938	C— 7	"

* G. stands for Guernsey, H. for Holstein.

serum treatment if we take into account certain facts. Though placed with their respective groups, one calf (No. 948) of the first group and one (No. 951) of the third group passed through the test considerably later than the others. With these was a colostrum-fed calf (No. 949) as a control. These all died. Some undetermined factors at this time increased the virulence of scours or reduced the resistance of the three calves, since this control calf is the only one of thirteen colostrum-fed calves to die. The significance of the data will be more impressive if we review the main facts of the preceding article.[1]

When colostrum is withheld the body becomes flooded with *Bacillus coli* types. Other forms, such as enterococcus, also appear but they are overgrown in cultures by the more numerous and very vigorous *Bacillus coli* types. After a calf has ingested colostrum or has been treated with serum, the invasion of the body is suppressed. The digestive tract, however, may not have been protected sufficiently to prevent scours or acute diarrhea from appearing after 1 or 2 days. In fatal cases the spleen, liver, and kidneys are shown to be free from bacteria or nearly so. An examination of notes made during the past 5 years on fatal spontaneous scours in colostrum-fed calves shows that with rare exceptions in these cases also there is no *Bacillus coli* invasion of the tissues. In the few animals in which *Bacillus coli* was abundant in such organs as the spleen, liver, and kidneys, the autopsy notes showed that the calf had not suckled the dam at all.

In those cases of fatal scours in which colostrum was found in the fourth stomach there was either incomplete protection of the intestinal tract because the colostrum was ingested late or else the digestive mechanism itself was imperfect.

Among the serum-treated calves which survived and were kept 2 to 8 weeks before being killed, joint lesions were practically absent. Occasional transient lameness was noted which may have been due to temporary localization of bacteria or injury. In two of the calves (Nos. 935 and 936) which received serum in the milk focal interstitial nephritis was found when they were killed at the age of 23 days.

CONCLUSIONS.

The serum of a normal lactating cow when injected into calves a few hours after birth saved only two out of five calves so treated.

Serum added to the milk of the first two meals saved three out of five. When the two methods were combined and the serum was both injected and fed all five calves so treated survived as normal calves. These figures to be significant should be compared with the controls of both series.[1] Since the beginning of this investigation twelve out of thirteen colostrum-fed calves have survived and only four out of fifteen from which colostrum has been withheld.

In those that died the serum whether fed or injected protected the internal organs against the invasion and multiplication of *Bacillus coli* and other intestinal types and in this respect its protective action is equivalent to that of colostrum in those calves which die of spontaneous scours.

THE IODINE CONTENT OF THE BLOOD FOLLOWING THYROIDECTOMY.

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The presence of iodine widely distributed in animal tissues has been well established. The occurrence of iodine in certain essential constituents of the thyroid gland has long been known, and there is evidence that the administration of iodine produces changes in the organ. Little is known concerning the relation of the thyroid to the iodine metabolism of the body.

Baumann[1] first showed that the thyroid gland contains iodine in amounts sufficient for quantitative determination, and obtained evidence that iodine forms an essential constituent of the gland. He found that the thyroids of twenty-six adults from Freiberg contained amounts of iodine varying from 0.22 mg. to 7.2 mg., the average iodine content of these twenty-six glands being 2.5 mg. The thyroids of four others contained 10.8, 14.9, 29.9, and 35.3 mg. respectively. Of thirty-four adults from Hamburg, the thyroids of thirty contained amounts of iodine varying between 0.3 and 9.7 mg. per gland, the average iodine content of the thirty glands being 3.83 mg. The thyroids of the other four individuals contained amounts of iodine in excess of 10 mg. per gland, the greatest amount being 27.6 mg. The thyroids of eleven adults from Berlin had an iodine content of less than 10 mg. per gland, the average content per gland being 6.6 mg. Two other thyroids contained 14.1 and 22.7 mg. respectively. Oswald[2] has also found a considerable variation in the average iodine content of the thyroid in different localities. Baumann found that the thyroids of children usually contained little or no iodine, and that the presence or absence of iodine and the amount, when present, varied according to the locality from which the glands were obtained. Wells[3] analyzed the thyroids of six children under 4 years of age from Chicago; three contained only traces and the other three contained from 0.011 to 0.092 per cent of iodine.

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[1] Baumann, E., *Z. physiol. Chem.*, 1895–96, xxi, 319; 1896–97, xxii, 1.

[2] Oswald, A., *Z. physiol. Chem.*, 1897, xxiii, 265.

[3] Wells, H. G., *J. Am. Med. Assn.*, 1897, xxix, 954.

Marine and Williams[4] and Marine and Lenhart[5] have arranged a series of thyroid glands from minimum to maximum in accordance with their iodine content and have found that they are at the same time arranged in series according to their histology, their colloid content, and their weight. In other words, the weight of the glands varied directly with the degree of hyperplasia and inversely with the iodine content. Marine and Lenhart[6] have found that thyroid hyperplasia in fish in any stage is diminished by addition of iodine to the water; they state that hyperplasia may be similarly prevented by iodine.

Loeb[7] observed hyperplasia in the thyroids of guinea pigs following the removal of part of the gland. He thought that there was evidence of a certain quantitative relation between the degree of hyperplasia and the amount of thyroid removed. Iodine did not diminish the intensity of the hyperplastic changes and he thought indeed that there was some indication that preparations of iodine might increase hyperplasia. Feeding of thyroid preparations, on the contrary, prevented hyperplasia.

Barell[8] found iodine in the ovaries, adrenal, and spleen of swine and cattle and in various commercial preparations of these organs. Howald[9] says that under normal conditions iodine is not found in human hair, dog's hair, or feathers of roosters; but following the administration of iodides the hair and feathers contain iodine. There was no iodine in the hair of a cretin 4 years of age, or of a woman with an extensive carcinoma of the thyroid.

Bourcet[10] has studied the distribution of iodine in the tissues of the rabbit. His figures obtained by examination of three rabbits are as follows: 200 gm. of blood contained 0.005 mg. of iodine; 60 gm. of heart, 0.0005 mg.; 400 gm. of liver with bile ducts, 0.71 mg.; 82 mg. of kidney, 0.027 mg.; 400 gm. of fat, none; 50 gm. of hair, 0.9 mg.; 500 gm. of muscle, 0.025 mg.; 40 gm. of lung, 0.03 mg.; 30 gm. of brain, 0.012 mg.; 10 gm. of pancreas, none; 200 gm. of skin without fur, 0.12 mg. Similar results were obtained with the tissues of a dog, iodine being recovered from the thymus, pituitary, and medullary tissue of the brain, mammary gland, and gravid uterus. Bourcet believes that all of the glands of the body contain iodine, though the amount is much less than that which is present in the thyroid. Iodine is excreted through the skin, the hair being the chief agent of elimination. Gley and Bourcet[11] find that the blood of dogs contains between 0.013 and 0.06 mg. of iodine per liter, which they think is in combination with protein.

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[4] Marine, D., and Williams, W. W., *Arch. Int. Med.*, 1908, i, 349.

[5] Marine, D., and Lenhart, C. H., *Arch. Int. Med.*, 1909, iv, 440.

[6] Marine, D., and Lenhart, C. H., *J. Exp. Med.*, 1911, xiii, 455.

[7] Loeb, L., *J. Med. Research*, 1919, xl, 199; 1919–20, xli, 485; 1920–21, xlii, 77.

[8] Barell, E., *Chem. Centr.*, 1897, lxviii, pt. 1, 608.

[9] Howald, W., *Z. physiol. Chem.*, 1897, xxiii, 209.

[10] Bourcet, P., *Compt. rend. Acad.*, 1900, cxxxi, 392.

[11] Gley, E., and Bourcet, P., *Compt. rend. Acad.*, 1900, cxxx, 1721.

Rogoff[12] found in two dogs that blood coming from the hyperplastic thyroid glands which had a very low iodine content did not cause the developmental changes which occur in tadpoles fed with thyroid. Blood of a third dog obtained during massage of the gland or during sympathetic stimulation produced the characteristic developmental changes. The samples of blood from this dog contained less than 0.005 mg. of iodine per gm. of dried blood. Later Rogoff and Goldblatt[13] used specimens of blood obtained from the glands of fifteen individuals on whom thyroidectomy had been performed. In this group were included instances of exophthalmic goiter, colloid goiter, and adenoma of the thyroid. They obtained no evidence of the presence of the active principle of the thyroid gland when these specimens of blood were fed to tadpoles. Examination of the specimens when sufficient amounts of blood were available failed to show the presence of iodine. Kendall[14] has separated the iodine of the thyroid into two parts. Part A is soluble in alkaline media and produces the same effects as thyroid when administered to patients; Part B is soluble in acids and does not produce the reactions of thyroid when administered to patients. When Marine and Feiss[15] perfused thyroids with solutions of potassium iodide they found that iodine was quickly taken up and fixed and was not readily removed by washing the gland with Ringer's solution. They obtained the same results by injecting the iodine into the circulation. Although, according to Marine and Rogoff,[16] iodine is quickly fixed in the thyroid for a time, it does not alter the influence of the thyroid tissue on the metamorphosis of tadpoles, but after several hours this property is distinctly increased. They regard the fixation of iodine and the formation of metamorphosis-promoting substance as distinct processes and think that the formation of this substance is dependent upon the amount of mother substance present in the gland rather than upon the amount of iodine, provided, however, that iodine is present in the usual quantity.

EXPERIMENTAL.

Observations recorded above concerning the relation of iodine to the thyroid gland have suggested the possibility that the thyroid might modify the iodine content of the blood. A quantitative study of the iodine in the blood has seemed desirable. Kendall[17] has

[12] Rogoff, J. M., *J. Pharmacol. and Exp. Therap.*, 1918–19, xii, 193.

[13] Rogoff, J. M., and Goldblatt, H., *J. Pharmacol. and Exp. Therap.*, 1921, xvii, 473.

[14] Kendall, E. C., *J. Biol. Chem.*, 1915, xx, 501.

[15] Marine, D., and Feiss, H. O., *J. Pharmacol. and Exp. Therap.*, 1915, vii, 557.

[16] Marine, D., and Rogoff, J. M., *J. Pharmacol. and Exp. Therap.*, 1916, viii, 439; 1916–17, ix, 1.

[17] Kendall, E. C., and Richardson, F. S., *J. Biol. Chem.*, 1920, xliii, 161.

recently published a method for determining iodine in the blood; this method is in reality a modification of his original[18] method of determining iodine in the thyroid gland. With this method, suitable for the determination of very small amounts of iodine, I have first determined the iodine content of the blood of normal dogs kept under laboratory conditions and then removed the thyroids of these animals in order to ascertain whether the iodine content of the blood is changed.[19] Determinations of the iodine in the blood have usually been made in duplicate by Kendall's method; occasionally in order to economize blood single determinations have been made at short intervals. After removal of a thyroid gland the animal has been kept under conditions identical with those before operation. The experiments are described in the order in which they were performed.

Determinations of the iodine in the blood of seventeen dogs have been made before the removal of the thyroid. The lowest amount of iodine found in normal blood is 0.0029 mg. in Experiment 10 and the highest amount is 0.0145 mg. in Experiment 8. The average amount of iodine in the blood of the seventeen dogs before the removal of the thyroid is 0.0079 mg. per 100 cc. of blood. Kendall gives 0.013 mg. of iodine as the average iodine content of the blood. He does not state whether or not the blood was human and does not give its source.

Experiment 1.—No determination of iodine in the blood was made before operation.

June 19, 1921. Both lobes of the thyroid were removed, a small tab being left at each lower pole with the parathyroids.

July 19. 100 cc. of blood contain 0.039 mg. of iodine.
100 cc. of blood contain 0.0383 mg. of iodine.

Oct. 3. 100 cc. of blood contain 0.03 mg. of iodine.
100 cc. of blood contain 0.0283 mg. of iodine.

Oct. 20. Animal killed. The small pieces of thyroid tissue at the lower poles are found to be very much enlarged and quite firm. Microscopic sections show active hyperplasia.

Experiment 2.—

July 16, 1921. 100 cc. of blood contain 0.0116 mg. of iodine.

Aug. 26. 100 cc. of blood contain 0.01 mg. of iodine.

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[18] Kendall, E. C., *J. Am. Chem. Soc.*, 1912, xxxiv, 894.

[19] All operations were performed under ether anesthesia.

Oct. 26. 100 cc. of blood contain 0.01 mg. of iodine.

Nov. 1. Both lobes of the thyroid are completely removed, leaving the parathyroid *in situ* at each lower pole.

Nov. 7. 100 cc. of blood contain 0.0141 mg. of iodine.
100 cc. of blood contain 0.014 mg. of iodine.

Nov. 9. The animal is found dead in its cage. At autopsy a large hematoma is found filling the space from which the left thyroid lobe had been removed. There is no suppuration of the wound.

Experiment 3.—

Aug. 25, 1921. 100 cc. of blood contain 0.0083 mg. of iodine.
100 cc. of blood contain 0.0066 mg. of iodine.

Sept. 3. The gland is removed completely, leaving the parathyroids at the lower poles *in situ*.

Sept. 17. 100 cc. of blood contain 0.02 mg. of iodine.
100 cc. of blood contain 0.0208 mg. of iodine.

The animal is very much emaciated and its hair is falling out in considerable quantities. It is killed with ether. The two parathyroids at the lower poles of the thyroid lobe are found intact.

Experiment 4.—

Sept. 15, 1921. 100 cc. of blood contain 0.0041 mg. of iodine.
100 cc. of blood contain 0.005 mg. of iodine.

Oct. 6. Both lobes of the gland are removed with the exception of a small tab at each lower pole.

Oct. 12. 100 cc. of blood contain 0.0166 mg. of iodine.
100 cc. of blood contain 0.0157 mg. of iodine.

Experiment 5.—

Sept. 20, 1921. 100 cc. of blood contain 0.0075 mg. of iodine.
100 cc. of blood contain 0.0083 mg. of iodine.

Oct. 4. Both lobes of the thyroid are completely removed.

Oct. 10. 100 cc. of blood contain 0.025 mg. of iodine.
100 cc. of blood contain 0.0216 mg. of iodine.

The animal is killed with ether. Its operative wound has healed by first intention.

Experiment 6.—

July 21, 1921. 100 cc. of blood contain 0.01 mg. of iodine.

Aug. 1. 100 cc. of blood contain 0.01 mg. of iodine.

Aug. 15. 100 cc. of blood contain 0.01 mg. of iodine.

Sept. 15. 100 cc. of blood contain 0.01 mg. of iodine.
100 cc. of blood contain 0.00833 mg. of iodine.

Oct. 21. Both lobes of the gland are removed, leaving the parathyroids at the lower poles *in situ*.

Oct. 30. 100 cc. of blood contain 0.03 mg. of iodine.
100 cc. of blood contain 0.0316 mg. of iodine.

The animal is killed with ether. The wound has healed by first intention.

Experiment 7.—

Oct. 19, 1921. 100 cc. of blood contain 0.0125 mg. of iodine.
100 cc. of blood contain 0.0133 mg. of iodine.

Nov. 8. Both lobes of the thyroid are removed, leaving the parathyroid at the lower pole *in situ*.

Nov. 13. 100 cc. of blood contain 0.025 mg. of iodine.
100 cc. of blood contain 0.0266 mg. of iodine.

Nov. 15. The animal is found dead in its cage. There was no clonic convulsion of tetany before death. There is slight suppuration of the wound at autopsy.

Experiment 8.—

Nov. 15, 1921. 100 cc. of blood contain 0.015 mg. of iodine.
100 cc. of blood contain 0.0141 mg. of iodine.

Dec. 1. The usual operation for removal of the thyroid is performed, leaving the parathyroid at each lower pole.

Dec. 3. 100 cc. of blood contain 0.0166 mg. of iodine.

Dec. 5. 100 cc. of blood contain 0.015 mg. of iodine.
There is slight suppuration of the wound.

Dec. 7. 100 cc. of blood contain 0.0066 mg. of iodine.
There is considerable discharge from the wound and the animal does not eat.

Dec. 9. 100 cc. of blood contain 0.0066 mg. of iodine.
The animal is killed with ether because its wound is suppurating and it does not eat.

Experiment 9.—

Dec. 3, 1921. 100 cc. of blood contain 0.00833 mg. of iodine.
100 cc. of blood contain 0.0075 mg. of iodine.

Dec. 3. The usual operation for removal of both lobes of the thyroid is performed, leaving a small tab of thyroid tissue with each parathyroid at the lower pole.

Dec. 5. 100 cc. of blood contain 0.02832 mg. of iodine.

Dec. 7. 100 cc. of blood contain 0.0375 mg. of iodine.

Dec. 8. The animal is found dead in its cage, symptoms of tetany having been present during 24 hours. Autopsy shows a small tab of thyroid tissue at each lower pole. These tabs of tissues are deep red in color. The parathyroids are not identified.

Experiment 10.—

Dec. 10, 1921. 100 cc. of blood contain 0.0033 mg. of iodine.
100 cc. of blood contain 0.0025 mg. of iodine.

Dec. 12. The usual operation for removal of both lobes is performed, leaving a small tab of thyroid tissue at each lower pole with the parathyroids.

Dec. 14. 100 cc. of blood contain 0.01 mg. of iodine.

Dec. 18. 100 cc. of blood contain 0.0116 mg. of iodine.
Following the operation this animal has eaten little. Immediately after removal of blood there are clonic convulsions of tetany and death occurred on Dec. 21.

At autopsy the wound is closed and there is no suppuration but the deeper tissues about the remaining bits of thyroid are red and indurated.

Experiment 11.—

Dec. 13, 1921. 100 cc. of blood contain 0.01 mg. of iodine.
100 cc. of blood contain 0.01 mg. of iodine.

Jan. 30, 1922. 100 cc. of blood contain 0.00833 mg. of iodine.

Feb. 1. The thyroid is removed as usual, leaving a small tab of thyroid tissue at each lower pole.

Feb. 6. The wound has healed by primary intention but there has been somewhat more edema than usual for several days. The animal has had today clonic convulsions of tetany. It is given 15 cc. of a 30 per cent solution of calcium lactate intraperitoneally and convulsions have disappeared within ½ hour.

Feb. 8. The calcium lactate is repeated because of clonic convulsions of tetany.

Feb. 11. 100 cc. of blood contain 0.015 mg. of iodine.
100 cc. of blood contain 0.02 mg. of iodine.

The animal has lost much weight and its hair is falling out in considerable quantities. Eats little.

Feb. 16. The animal is found dead in its cage. No suppuration of the wound is observed at autopsy. The bits of thyroid tissue at each lower pole are embedded in scar tissue.

Experiment 12.—

Dec. 18, 1921. 100 cc. of blood contain 0.0066 mg. of iodine.
100 cc. of blood contain 0.0066 mg. of iodine.

Dec. 23. The thyroid is removed, leaving the parathyroids at each lower pole.

Dec. 27. 100 cc. of blood contain 0.011 mg. of iodine.

Dec. 29. 100 cc. of blood contain 0.0097 mg. of iodine.

Dec. 31. 100 cc. of blood contain 0.00833 mg. of iodine.

The animal died following the removal of blood. At autopsy no suppuration of wound is found.

Experiment 13.—

Oct. 28, 1921. 100 cc. of blood contain 0.005 mg. of iodine.
100 cc. of blood contain 0.00583 mg. of iodine.

Jan. 10, 1922. The thyroid gland is removed.

Jan. 14. 100 cc. of blood contain 0.0233 mg. of iodine.
100 cc. of blood contain 0.02 mg. of iodine.

Jan. 15. The animal is found dead in its cage. No suppuration of the wound is found at autopsy.

Experiment 14.—

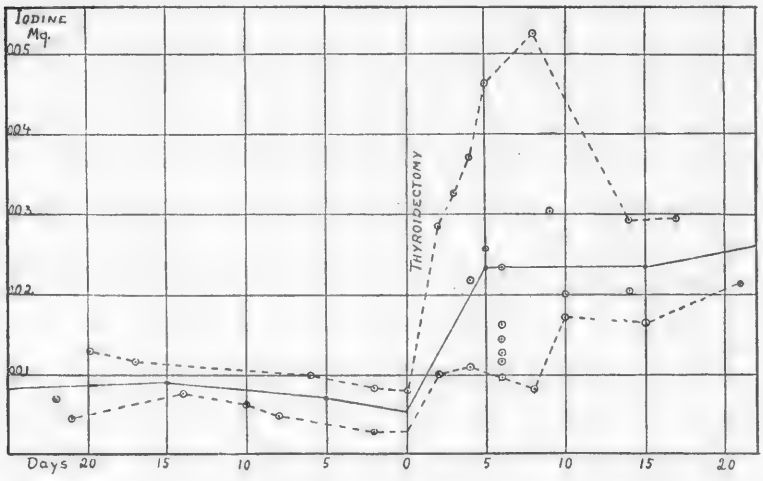
Jan. 3, 1922. 100 cc. of blood contain 0.0055 mg. of iodine.
100 cc. of blood contain 0.0055 mg. of iodine.

Jan. 11. The thyroid is removed.

Jan. 14. 100 cc. of blood contain 0.0333 mg. of iodine.
100 cc. of blood contain 0.036 mg. of iodine.

The animal died while being bled. No suppuration of the wound is found at autopsy.

This group of experiments demonstrates that the amount of iodine in the blood is greatly increased after thyroidectomy (Text-fig. 1). This increase is seen in all experiments. In Experiment 8 the increase is small and the amount of iodine in the blood soon falls below that found before operation; but this dog has suffered from a badly infected wound and did not eat, so that its store of iodine has not been re-



TEXT-FIG. 1. Composite chart of iodine determinations made before and after thyroidectomy. Maximum and minimum determinations are connected by broken lines to show the degree of variation. Only one determination was made on the day of operation, since it seemed undesirable to draw blood at this time. The date of operation has been crossed by the minimum line at the level of the last determination. The continuous line is an average of all determinations made within a period of 10 days, the date of operation being crossed midway between the minimum and maximum lines.

plenished. Increase of iodine in the blood varies but in a number of experiments is considerable. For example, in Experiment 14 there is an increase after removal of the thyroid from 0.0055 mg. to 0.033 mg. in 100 cc. of blood, and in Experiment 13 the increase is from 0.00583 mg. of iodine before operation to 0.0233 mg. per 100 cc. of blood after thyroidectomy. These variations are too great to be explained by experimental error, for Kendall has shown that there is a constant

loss of about 3 per cent of iodine determined by his method. The change noted after operation cannot be due to difference in the iodine content of the food, for in a number of instances repeated determinations before operation when the food was identical with that received after operation show little variation. For example, Experiment 6 shows a variation from 0.01 mg. to 0.00833 mg. among iodine determinations made on four occasions between July 21 and September 15; during this time the dog received the same diet as after the operation so that an increase from 0.01 mg. to 0.03 or 0.0316 mg. of iodine per 100 cc. of blood within the first 10 days after operation cannot be explained by variation of the iodine in the food.

It is noteworthy that the increase of iodine occurs soon after removal of the thyroid gland; in Experiment 9 there is an increase from 0.00833 mg. to 0.02832 mg. of iodine per 100 cc. of blood, and in Experiment 10 from 0.0033 mg. to 0.01 mg. within the first 48 hours after removal of the thyroid gland. If this sudden increase in the iodine content of the blood were due to injury of the gland during the operation the iodine content of the blood would return to normal in a very short time, but in Experiment 5, 6 days after operation the iodine content of the blood is 0.025 mg. per 100 cc. of blood, or three times that before operation, and in Experiment 3, 14 days after operation it is 0.0208 mg. as compared with 0.0083 mg. before operation; in Experiment 1, 30 days after operation the iodine content of the blood is 0.0386 mg. and 106 days after operation it is 0.0291 mg. per 100 cc. of blood.

The possibility suggests itself that the thyroid influences the metabolism of iodine in much the same way that the pancreas controls the metabolism of glucose. Present knowledge of the subject does not establish any close analogy between iodine and carbohydrate metabolism.

A series of experiments has been performed to determine the effect of thyroid feeding on the iodine content of the blood of dogs of which the thyroids had been removed. Following the operation determinations of the iodine content of the blood have been made and the dogs have then been fed with fresh thyroid of sheep in addition to the usual food. After a number of days, the iodine content of the blood has again been determined. The thyroid feeding has been discontinued and later iodine determinations have been made.

Experiment 15.—

July 15, 1921. 100 cc. of blood contain 0.01 mg. of iodine.

Aug. 25. 100 cc. of blood contain 0.0125 mg. of iodine.

Oct. 15. 100 cc. of blood contain 0.0116 mg. of iodine.

Nov. 1. Both lobes of the gland are removed, the parathyroids at the lower pole being identified and left *in situ*.

Nov. 11. The wound has healed by first intention.
100 cc. of blood contain 0.02 mg. of iodine.
100 cc. of blood contain 0.02 mg. of iodine.

Nov. 22. 100 cc. of blood contain 0.0233 mg. of iodine.
100 cc. of blood contain 0.0233 mg. of iodine.

Jan. 5, 1922. 100 cc. of blood contain 0.021 mg. of iodine.
100 cc. of blood contain 0.021 mg. of iodine.

Following the removal of blood the feeding of fresh thyroids of sheep was started. The dog received from two to four lobes each day.

Jan. 19. 100 cc. of blood contain 0.01 mg. of iodine.
100 cc. of blood contain 0.012 mg. of iodine.

Experiment 16.—

Dec. 13, 1921. 100 cc. of blood contain 0.0066 mg. of iodine.
100 cc. of blood contain 0.0066 mg. of iodine.

Dec. 20. The thyroid is removed, leaving the parathyroid at each lower pole.

Dec. 25. 100 cc. of blood contain 0.0483 mg. of iodine.

Dec. 28. 100 cc. of blood contain 0.0566 mg. of iodine.

Jan. 3, 1922. 100 cc. of blood contain 0.0333 mg. of iodine.

Jan. 6. 100 cc. of blood contain 0.0333 mg. of iodine.

The feeding of fresh thyroid of sheep was started. The animal receives from five to eight lobes each day.

Jan. 19. 100 cc. of blood contain 0.0066 mg. of iodine.
100 cc. of blood contain 0.0075 mg. of iodine.

Jan. 20. The thyroid feeding is discontinued.

Feb. 14. 100 cc. of blood contain 0.0216 mg. of iodine.
100 cc. of blood contain 0.0233 mg. of iodine.

The animal is killed with ether following the removal of blood.

Experiment 17.—

Dec. 29, 1921. 100 cc. of blood contain 0.007 mg. of iodine.
100 cc. of blood contain 0.007 mg. of iodine.

Jan. 20, 1922. The thyroid is removed, leaving a very small tab of thyroid tissue at each lower pole with the parathyroid.

Jan. 27. The animal is losing its hair in considerable quantities.

The feces are very hard, dry, and powdery. The wound has healed by first intention.

Feb. 4. 100 cc. of blood contain 0.0166 mg. of iodine.
100 cc. of blood contain 0.0168 mg. of iodine.

Feb. 9. The feeding of fresh thyroid of sheep is started, the animal receiving from six to eight lobes each day.

Feb. 15. 100 cc. of blood contain 0.0066 mg. of iodine.

The hair has stopped falling out and the animal has somewhat softer feces.

Mar. 1. The feeding of fresh thyroid is discontinued.

Mar. 20. The hair is falling out in considerable quantities, and the feces are hard and powdery.

100 cc. of blood contain 0.015 mg. of iodine.

Experiment 18.—

Feb. 1, 1922. 100 cc. of blood contain 0.00583 mg. of iodine.

100 cc. of blood contain 0.0066 mg. of iodine.

Feb. 11. The thyroid is completely removed, leaving only the parathyroids at each lower pole.

Feb. 17. 100 cc. of blood contain 0.0133 mg. of iodine.

100 cc. of blood contain 0.0116 mg. of iodine.

The hair is falling out in considerable quantities and the animal is losing weight. There are mild clonic convulsions of tetany.

Feb. 19. The feeding of fresh thyroid is started, the animal receiving from four to eight lobes each day.

Feb. 23. The hair has stopped falling out and the animal is gaining in weight. There are no symptoms of tetany.

100 cc. of blood contain 0.008 mg. of iodine.

The thyroid feeding is discontinued.

Mar. 5. The animal is losing weight, eats very little, and the hair is falling out.

100 cc. of blood contain 0.0125 mg. of iodine.

The animal is killed with ether. The parathyroids are found embedded in scar tissue at each lower pole.

In this group of experiments the foregoing results are confirmed and further evidence that increase of the iodine content of the blood is due to the removal of the thyroid is obtained. Feeding of fresh thyroid of sheep to thyroidectomized dogs reduces the iodine content of the blood so that it approximates the preoperative level. In Experiment 15 the iodine content of the blood before operation was 0.0125 mg. per 100 cc., and on the day the animal first received fresh thyroid with its food the postoperative iodine of the blood was 0.021 mg.; after 14 days of thyroid feeding the iodine content of the blood had fallen to 0.012 mg.

In Experiment 16 the preoperative iodine content of the blood was 0.0066 mg. with a postoperative iodine content which reached the height of 0.0566 mg. and was still 0.033 mg. after the removal of 400 cc. of blood within a period of 12 days. 13 days after thyroid feeding

had been started the iodine content of the blood was 0.0075 mg. In Experiments 17 and 18 the iodine content of the blood rose again to the former postoperative level when the thyroid feeding was discontinued.

When some substance which the thyroid gland normally supplies is lost as the result of removal of the organ, the iodine content of the blood increases to an abnormal level. This hypothetical substance may be restored by feeding thyroid, and under this influence the normal iodine level is restored. Iodine in the blood decreases even though the thyroid gland is no longer present to remove it from the blood. The thyroid supplies some substance which profoundly influences the metabolism of iodine not only within the thyroid but elsewhere in the body.

CONCLUSIONS.

1. After thyroidectomy in dogs the iodine content of the blood is increased.

2. The administration of fresh thyroid gland of sheep by mouth to thyroidectomized dogs causes the iodine content of the blood to fall so that it returns to the normal preoperative level; when the thyroid feeding is discontinued the iodine content of the blood is again increased.

It is with pleasure that I acknowledge the advice and encouragement given by Professor E. L. Opie.